A primer on cloning in *E. coli*

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Things to add:

gel electrophoresis methods
basic bacteriology methods

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A primer on cloning in *E. coli*

Overview

"Cloning" as a molecular biology term refers to the manipulation of small DNA fragments to create DNA assemblies that can be used to express RNAs and protein coding genes in organisms. *E. coli* is often used because of the ease of manipulating DNA using this bacteria. Specifically, *E. coli* is simple to grow, grows rapidly, can reliably be transformed with DNA and is highly efficient at amplifying plasmid DNA allowing for rapid production of even milligram quantifies of DNA.

The techniques used in DNA molecular biology are limited in number and relatively easy to perform. They include basic bacteriology to grow *E. coli* and basic biochemistry to manipulate DNA. Specifically, they include production of competent cells for introducing DNA into *E. coli*, isolation of plasmid DNA, restriction enzyme cleavage of DNA, gel electrophoresis, polymerase chain reaction amplification of DNA, and several methods for assembly of DNA fragments into plasmids. An overview of restriction enzymes and PCR is provided for those unfamilar with these techniques.

E. coli vectors

Types of *E. coli* vectors

The most commonly used *E. coli* vectors are called plasmids. These supercoiled circular DNAs (ranging from ~ 2 kb to ~25 kb) are maintained in *E. coli* at medium to high copy number. Cosmids[1] (maintained in *E. coli* in medium copy) and fosmids[2] (maintained in *E. coli* in single copy) are plasmid-like vectors that hold larger inserts up to around 40 kb in length. To manipulate larger fragments of DNA up to 350 kb in size, bacterial artificial chromosomes (BACs) are used. This primer focuses on construction of plasmids.

Components of typical plasmid vectors

All plasmids contain several elements that allow them to be maintained and manipulated in *E. coli*.

Origin of replication

All plasmids have an origin which is required for the plasmid to be replicated in *E. coli* during cell division. The vast majority of plasmids used in *E. coli* contain an origin derived from pUC plasmids, which is a mutant version of a pBR322 origin. This origin keeps plasmid copy very high (usually >500 copies per cell). The advantage of this is that they provide great yields of plasmid DNA. The disadvantage is that some sequences which are toxic, recombine or are otherwise unstable, can be difficult to maintain in *E. coli* using these high copy plasmids.

Other origins with lower copy numbers exist. The larger pBR322 origin keeps plasmid copy number around 20-50, the P15A origin keeps copy number around 10-15 copies per cell, and the SC101 origin at around 5 copies per cell.

In addition to these origins, two others are worth noting. One is called the R6K origin. This origin requires a R6K plasmid encoded gene called *pir* to replicate. *E. coli* can be provided *pir in trans*. The plasmids carrying the R6K origin will replicate in *E. coli pir*$^{+}$ strains, but not in *E. coli* strains we standardly use.

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[1] Cosmids are based on a standard origin of replication, but unlike plasmids, they can be introduced into cells using lambda phage packaging. Cosmids were designed to get around the intrinsic size limitation of DNA transformation in *E. coli*. Larger DNA molecules transform less efficiently.

[2] Fosmids are similar to cosmids but are based on the F' origin of *E. coli* which limits these clones to single copy and makes them more stable.

The other origin worth noting is the F1 origin from the single stranded phage F1. Plasmids containing this origin can be induced to produce single strand DNA copies of the plasmid. These types of plasmids were highly popular in the 1980s and early 1990s before the advent of and wide spread use of PCR. At that time most DNA sequencing was performed through the synthesis of a second DNA strand in the presence of ddNTPs (Sanger sequencing) using a single stranded DNA template with an annealed oligonucleotide. pBluescript plasmids still commonly used today contain this origin.

Antibiotic resistance genes

The other key feature virtually all plasmids have is a gene encoding a resistance to an antibiotic that is used to select for plasmid maintenance in *E. coli*. There are multiple different resistance genes which work using different mechanisms.

Ampicillin

Ampicillin is a β-lactam antibiotic that acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make their cell wall. The most common resistant gene *bla* (also often labelled *ampR*) encodes β-lactamase which confirms ampicillin resistance by cleaving ampicillin. It is secreted into the periplasm of *E. coli*. Although technically bacteriolytic, in practice because β-lactamase is secreted, selection for plasmid containing cells is poor in liquid culture. Even, on agar plates, satellite colonies appear after the β-lactamase has lowered the concentration of ampicillin in the vicinity of the resistant colony. The typical working concentration for ampicillin is 50-100 µg/ml. Some investigators use carbenicillin in place of ampicillin because it is more stable, reducing the problem of satellite colonies and increasing selection in liquid culture.

Kanamycin

Kanamycin is an aminoglycoside that acts by binding to the 30S subunit of the bacterial ribosome and inhibiting translation. The *kanR* gene, derived from the bacterial transposon Tn903 is a bacterial aminoglycoside phosphotransferase, which inactivated kanamycin. Working concentration is 25-50 µg/ml.

Tetracycline

Tetracycline works by binding the *E. coli* ribosome and blocking protein synthesis. *tetA* encodes a membrane protein that pumps tetracycline out of cells.[3] Working concentration is 10-15 µg/ml.

Chloramphenicol

Chloramphenicol acts by binding the 50S subunit of the bacterial ribosome and inhibits translation. C*m*, the chloramphenicol acetyl transferase gene, confers resistance to chloramphenicol. Working concentration is around 12.5 µg/ml .

Other antibiotics

Zeocin, bleomycin, hygromycin B and the aminoglycoside spectinomycin are others antibiotics for which resistant genes have been incorporated into plasmids that are widely available for molecular biology.

Master Cloning Sites

Master cloning sites (MCS) are synthetic (not derived from natural sources) sequences that contain multiple restriction sites that have been introduced into cloning vectors to facilitate insertion of DNA fragments using a standard restriction enzyme approach.

LacZα

LacZα is a small peptide from the *LacZ* β-galactosidase gene that will complement deletions of *LacZ*α in the *E. coli* genome *in trans*. This fragment is encoded in many plasmids to provide “blue-white” screening

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[3] *tetA* is often mis labelled as *tetR*. However, note that *tetR* is formally a different gene encoding another protein used in molecular biology. This is a repressor that binds to the t*etA* promoter in the absence of tetracycline, but comes off to de-repress the *tetA* gene in the presence of tetracycline. Tetracycline inducible systems used in mouse genetics use the *tetR* repressor and operator sequences to control gene expression of mammalian genes in a tetracycline dependent manner.

for insertion of DNA into a plasmid (usually at a MCS). Insertion of a fragment into the MCS disrupts expression of *LacZ*α, and the resulting bacteria cannot make β-galactosidase, and stay white, instead of turning blue because they fail to cleave the β-galactosidase activity indicator X-gal. Using this system requires the recipient strain to contain the appropriate *LacZ* mutation (often Δ(lacZ)M15[4]) and to be have the operon inducible on plates lacking lactose. Typically, isopropyl β-D-1-thiogalactopyranoside (IPTG) is added to plates. IPTG acts as an inducer of the *lac* operon by binding *lacR*. This is necessary in cases where the *lacI*^{q} mutation is present, a mutation which overexpresses the lac repressor to provide better control over induction of the *lac* operon. Typically, 30 ul of 1M IPTG and 40 ul of 2 % X-gal are added to a plate at the time of plating the bacteria.

ccdB

The *ccdB* gene is a highly effective negative selection marker for *E. coli*. plasmid DNA containing a *ccdB* gene will not grow in most strains of *E. coli*, but can be grown in specialized strains. This can be a very effective strategy to select against clones not containing an insert (which replaces *ccdB)*. *ccdB* acts as a negative selection by inhibiting the function of *gyrA,* a DNA gyrase. Plasmids containing the *ccdB* gene are grown in strains resistant to the action of *ccdB*. DB3.1 and *ccdB* survival™ are two strains commonly used to grow *ccdB* gene containing plasmids[5].

[4] often written lacZ DM15

[5] DB3.1 contains a *gyrA462* mutant resistant to the toxic effects of *ccdB*. *ccdB* survival™ is resistant because it overexpresses ccdA which blocks the *ccdB* toxin. Note that *ccdA* is encoded on the F' factor and any F' positive *E. coli* will be resistant to *ccdB* including multiple popular *E. coli* strains such as NEB Stable, JM109, and XL1 Blue.

Design of DNA constructs

There are many factors that should be considered in designing a cloning experiment to both simplify and increase the efficiently of the methodology.

Choice of vector

DNAs that replicate in *E. coli* are called plasmids and also often referred to as vectors. In many cases the choice of vector can be very important for efficient long term progress in creating a series of constructs for a project. For example, one might desire to express the same gene product under the control of multiple different promoters to determine which tissue types are required for the expression of a phenotype. If the vector is chosen wisely and a long term approach is taken which orchestrates the different clones being created, the project can proceed much more rapidly than if a random vector is chosen. When deciding on which vector to use consider 1) whether or not you are likely to use the DNA fragment in many parallel experiments that require, distinct, but similar plasmids. If so you might consider using a Gateway style vector system. You should also consider the drug resistance marker on the plasmid. The efficiency of several of the common approaches used for cloning (such as Golden Gate cloning and Gibson assembly) benefit greatly from swapping antibiotic resistance markers.

Source of insert

As with the vector, the source of the insert DNA can have significant impact on the efficiency of progress and the level of certainty that one can have about the exact sequence of the clone that has been created. Plasmids, endogenous sources of genomic DNA, Polymerase Chain Reaction (PCR) products, first strand synthesized cDNA and chemically synthesized DNAs are the most common potential sources of DNA.

Plasmids

If a previously sequenced plasmid clone of the desired DNA insert is available this is usually the safest approach to take. However, in many cases restriction endonuclease sites for cloning are not positioned in appropriate positions or even present in the DNA of interest. Thus, a PCR is often used in combination with a well characterized clone to synthesize a fragment of interest in order to move it into another vector of interest.

Genomic or cDNA

Another common source of DNA is genomic DNA or cDNA (reverse transcribed RNA[6]) derived from an organism. When using a genomic source of DNA one must consider the possibility that the sequence of the gene from the source may not be identical to the “reference sequence” being analyzed *in silico*. It is important to keep in mind that sequences can vary significantly from strain to strain in many species. Even the coding region of genes can have 3rd base synonymous changes. Promoter, introns and 3' UTRs can have dramatic differences including deletions and insertions and many bp changes[7]. These can interfere with PCR when choosing primers in upstream, downstream, and intronic sequences.

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[6] Reverse transcription is making a DNA copy of an RNA. Usually, mRNA is mixed with an oligo(dT) primer and dNTPs and a reverse transcriptase is used to synthesize a DNA copy of the RNA.

[7] Individual to individual sequence polymorphisms differ greatly by organism. For example, *C. elegans* is virtually clonal and has no variation. In mouse, there is significant variation between strains, but specific inbred stains have little variation. But, for organisms like zebrafish extensive individual to individual variation is found.

Synthetic DNA

In recent years synthetic DNA blocks from 100 bp - 3 kb (and increasingly larger) have become a reasonably priced alternative[8]. Especially in cases where one might wish to alter codon usage[9] to improve expression of a foreign gene, this is a great option.

Overview of different approaches to creating clones

Restriction enzyme based cloning

This time tested method is efficient, reliable, simple and usually inexpensive. A vector is cleaved with one or more **restriction enzyme(s)** and one or more insert DNA fragment(s) with cohesive ends is ligated together *in vitro* (Figure 1) then reintroduced into bacteria.

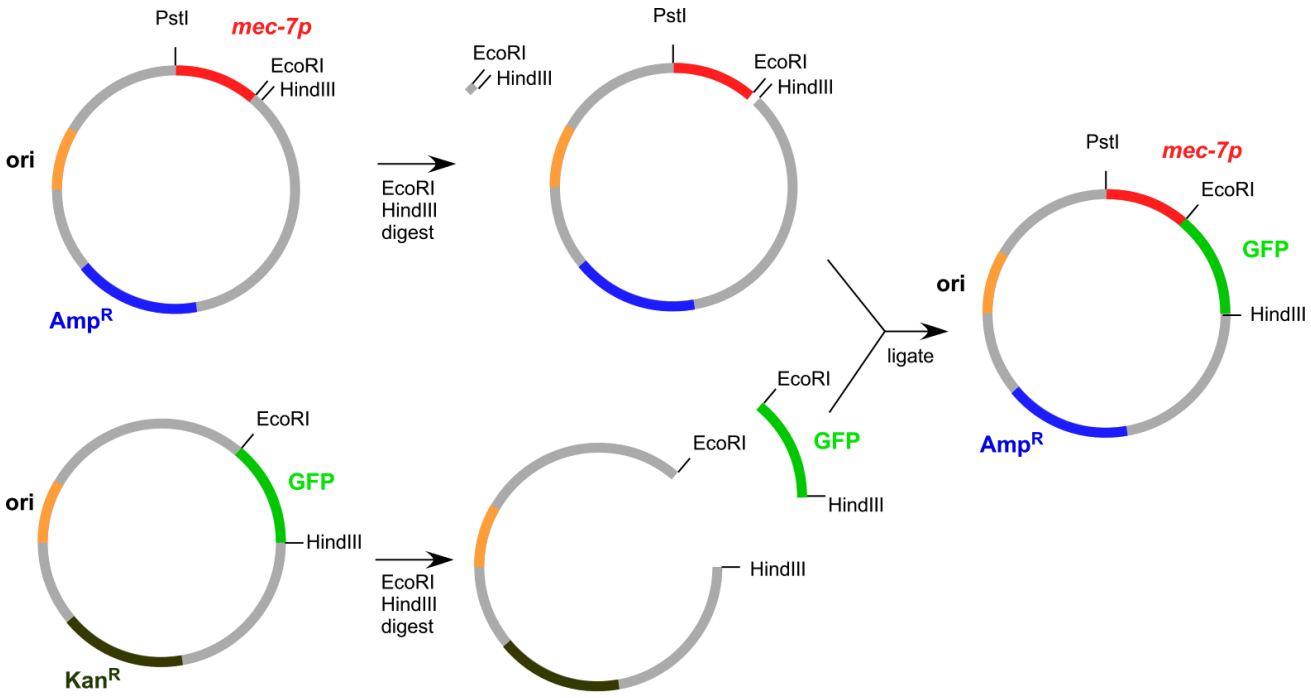


Figure 1. A simple schematic for sub-cloning of a DNA fragment from one plasmid into another plasmid using traditional restriction enzyme cloning.

When executed appropriately fragments as large as 15-20 kb in size can be inserted into a vector with the final plasmid size approaching 25 kb. The major limitation of this approach is the availability of restriction sites in the vector and insert. In the case of the insert, the appropriate sites can often be introduced using a PCR to synthesize the fragments being inserted. Many vectors have MCS (master cloning sites) with multiple

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[8] as of June 2019, Twist Bioscience will synthesize 0.3 to 1.8 Kb fragments for $0.07 per base in under two weeks.

[9] Most amino acids can be coded by several different codons. Since different organisms have different GC to AT compositions, different codons often occur at different frequency in different organisms, and most highly expressed gene utilize the more abundant codons (and also express higher levels of the tRNAs that carry those amino acids during translation. Often altering codons that are rarely utilized to ones that are more often utilized will increase expression. Thus, when one is expressing a foreign gene such as a fluorescent protein, different variations of the gene may codon usage optimized for different organisms.

restriction sites at the appropriate position in the vector permitting easy insertion of a wide variety of DNA fragments.

The general approach is to digest a vector DNA with restriction enzymes, and an insert DNA with the same enzymes, then ligate them together (Figure 1). The behavior of DNA in ligations can also greatly influence the cloning efficiency. Design of cloning strategies with these facts in mind can greatly increase the probability of success.

Single vs. double cut method

Because unimolecular ligation is favored over bimolecular reactions, designing the vector such that it cannot efficiently re-ligate in a unimolecular reaction will greatly increase the success rate. Using two distinct restriction enzymes also forces the orientation of the insert in the resulting clones (Figure 2).

Blunt vs "sticky" restriction sites

Some restriction enzymes leave blunt 5' and 3' ends, while others leave a 5' or 3' overhang. These overhangs drive specificity of interactions between DNA molecules, while blunt ended DNAs interact non-specifically and will ligate with any other blunt fragment leading to greater flexibility in inserting a fragment, but at the cost of greatly reduced ligation (~20 fold less) efficiency. Using sticky A-sticky B or sticky-blunt, rather than blunt-blunt ends to will lead to both increased efficiency and force the orientation of DNA insertion.

When the only option is to insert a fragment into a single cut vector, de-phosphorylation[10] of the vector is an option, but this can be tricky to perform efficiently. Use a heat labile phosphatase like shrimp alkaline phosphatase, and remember that zinc is an essential co-factor for these enzymes.

Typical methodology for restriction enzyme cloning

A typical cloning experiment takes several days. Much of this time is simply waiting for bacterial growth. The first step can be done in 4-5 hrs. The final step of characterizing the clone also takes ~ 3- 4 hrs.

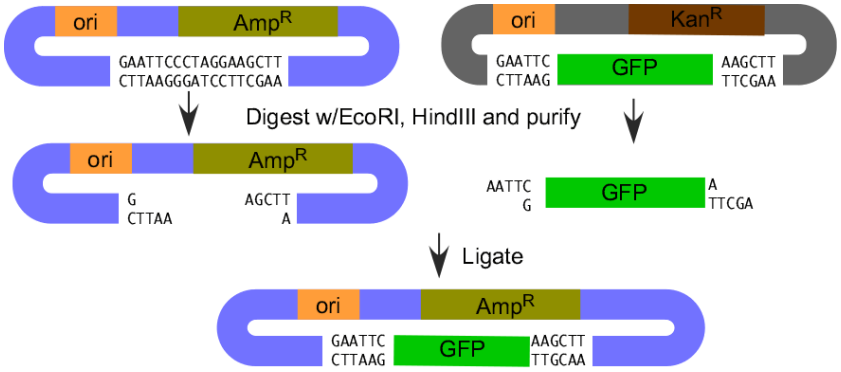


Figure 2. Nucleotide level view of standard double -sticky Restriction Enzyme subcloning experiment.

In separate tubes, ~ 200 ng of vector DNA is digested with one or more enzymes and ~200 ng of insert DNA is digested with one or more enzymes for one hr. The vector and insert DNAs are purified by gel electrophoresis, or using DNA binding columns. This takes ~ 30 minutes.

DNAs are ligated for 30 minutes (this can be extended overnight) and the ligated DNA is transformed into *E. coli* and the cells are plated on agar plates with the appropriate antibiotic for selection. The bacterial colonies grow up in 12-16 hrs.

Individual colonies are then grown up overnight in liquid culture, and plasmid DNA is isolated and analyzed (usually by restriction digestion) to identify clones of the correct structure.

[10] Ligation of DNA requires a 5' PO_4. Removing the phosphates from the ends of the vector, but not the insert, allows one phosphate bond to be form when an insert is ligated into the vector. E. *coli* ligates the remain 'nick' after transformation.

Golden Gate style cloning

Golden Gate assembly is a derivative of standard restriction enzyme cloning. This methods was originally described by Marillonnet (Engler et al., 2008; Engler and Marillonnet, 2014) and was made popular and its power highlighted by the Golden Gate TALEN method devised by the Bogdanove and Voytas lab (Cermak et al., 2011). The term Golden Gate cloning was first defined by Engler et al. 2008, for being a fast, one-tube reaction that yielded close to 100% correct clones. The method takes advantage of a small set of type IIS restriction enzymes that cut at asymmetric recognition sequences AND cut outside of the recognition motif (Figure 3). This method permits building complex clones assembled from one vector and multiple distinct insert fragments (sometimes as many as 10-20). The method works exceedingly well, but does have limitations. The guiding principle behind the approach is that the final product does not contain the recognition site of the enzyme used for cloning. The vector has restriction sites contained in the spacer eliminated in the cloning, and the inserts have sites on the edges of the fragments being inserted. Because these enzyme cut with overhangs of 3 or 4 base pairs that can be any sequence, a set of overlapping fragments each with distinct sticky ends can be assembled with the fragments theoretically only being able to assemble in one specific order. Furthermore, because the final product does not contain sites, a ligation-digestion cycle can be performed repeatedly, re-digesting products that assemble incorrectly without effecting properly assembled fragments, greatly increasing the efficiency of these reactions. Why not use this all the time? Because, a significant limitation is that the fragments cannot contain the restriction site! Since these enzyme cleave at a 6 or 7 base recognition sites, these sites are found at between ~ 1 in 2000 to 1 in 8,000 bases in typical sequences. If sites are absent, or engineered out of inserts, this is an efficient method for creating complicated multi-insert clones.

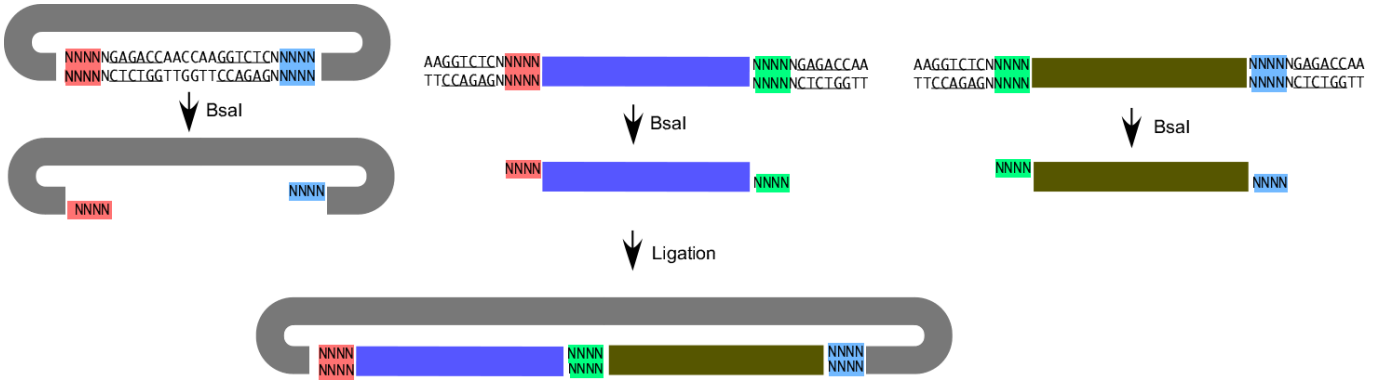


Figure 3 A diagram of Golden Gate type cloning using a plasmid as a vector, and two PCR products as inserts. Note that the reaction can also be performed with all plasmids, or even all PCR fragments. The red green and blue NNNN represent different 4 bp sequence motifs.

Typical methodology for Golden Gate cloning

A single tube reaction containing ~ 50 ng of each insert DNA (either as preexisting plasmids or purified PCR fragments), 50 ng of vector DNA and a type II restriction enzyme and T4 DNA ligase is assembled and cycled between rounds of 37°C incubation and 15°C incubation to repeatedly favor digestion, then ligation. If all of the reagents are in hand, the reaction only takes a few hrs to perform. The resulting reaction is then directly transformed into *E. coli* and plated on petri dishes containing the appropriate antibiotic containing media. DNA is isolated from the resulting colonies and analyzed (usually by restriction digestion) to identify insert containing plasmids.

Gateway cloning

Gateway cloning is a specialized form of cloning developed in the late 1990's by Life Technologies, Inc. and subsequently acquired by Invitrogen (Hartley, 2000). It uses lambda phage site-specific recombinases to catalyze *in vitro* recombination between distinct plasmids (or linear DNA fragments) carrying derivatives of the attB, attP, attL, and attR sites.

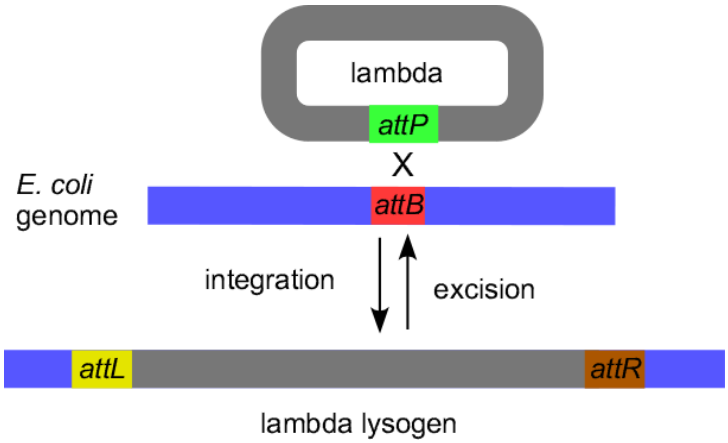


Figure 4. Lambda integration and excision from the *E. coli* genome form the basis for Gateway cloning.

Two different enzymes catalyze the forward and backwards reaction of lambda phage integration and excision into a specific 34 bp site in the *E. coli* genome (Figure 4). The Gateway strategy (Figure 5) involves two steps. The first involves inserting linear fragments flanked by attB sites into a "Donor" plasmid that contain attP sites flanking a negative selection *ccdB* gene. This yields "Entry" clones which contain the insert flanked by attL sites. These clones are in turn recombined with "Destination" vectors to create the final clones of interest. The Destination vector contains attR sites flanking a negative selection gene *ccdB*. The Donor and Destination vectors containing *ccdB* are created and amplified in special strains that are resistant to the killing effects of the *ccdB* gene. Once the Destination vectors and Entry vectors have been created, simply mixing of two plasmids with the appropriate recombinase enzyme catalyzes the recombination between the two plasmids to create the final desired product. Derivatives of this method which use several different flavors of *att* sites that allow for the insertion of multiple different fragments into a destination vector similar to Golden Gate cloning (Figure 6).

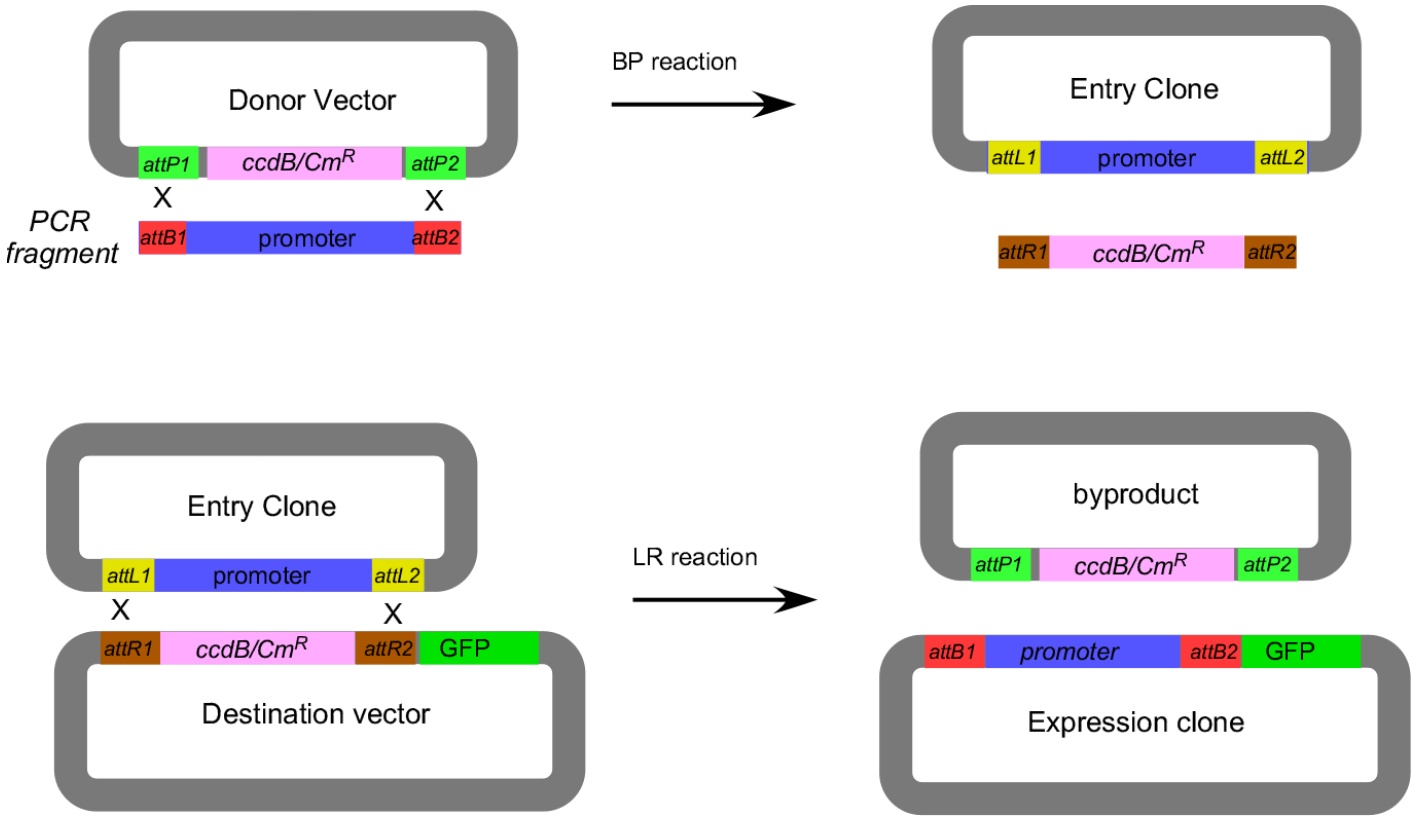


Figure 5. Diagrams of the recombination events in BP and LR reactions of the Gateway cloning system.

The advantages of this system include 1) that the system is modular and fast (once the parts have been built). The disadvantages are 1) that there is a 34 bp *attB* site between each junction in the final product, 2) that the destination vectors requires special competent cells of *ccdB* resistant *E. coli*, 3) that these *ccdB* vectors are somewhat unstable and have a tendency to delete out the *ccdB* cassette, 4) that the reaction mixes are expensive to purchase. Although popular in the last 10 years, the advent of Gibson assembly has greatly limited the benefit of this method.

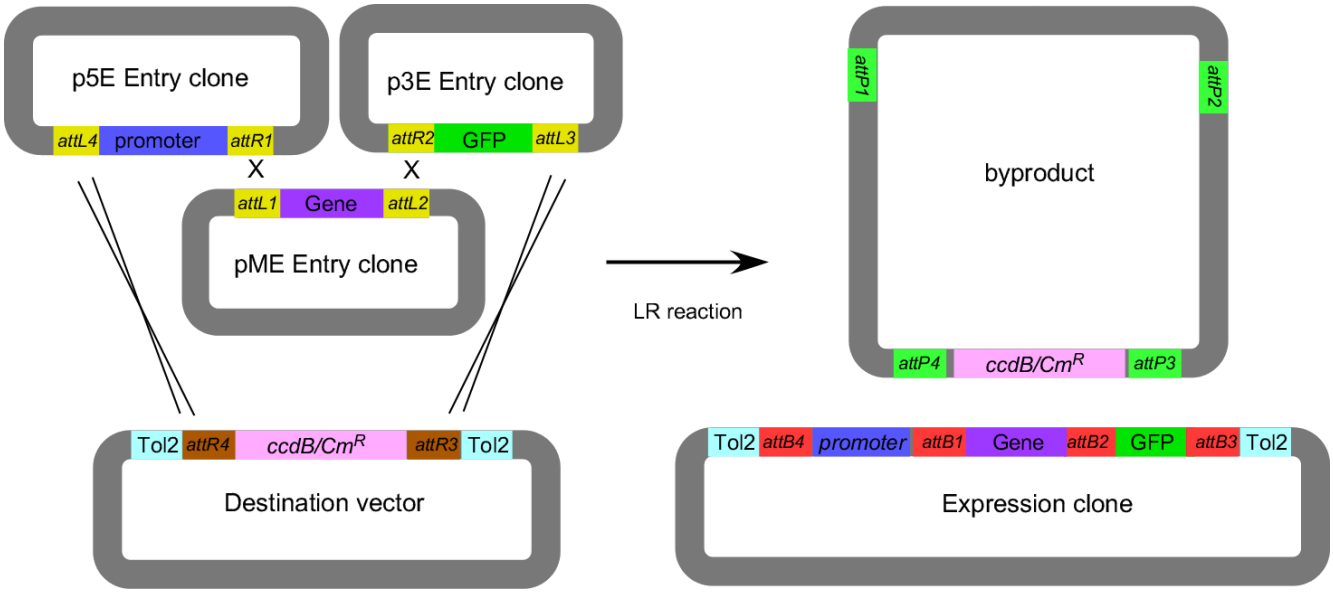


Figure 6. Multi-insert cloning using the Gateway cloning system. Different sets of *attL* and *attR* sites that will not recombine with other *attL* and *attR* sites allow for multiple inserts.

Typical methodology for Gateway cloning

If all of the component clones are already built, one simply mixes ~ 100 ng of each plasmid, adds the LR clonase mix, incubates an hr at 25°C, and incubates briefly with proteinase K to digest the enzymes, and transforms *E. coli*. The reaction can easily be performed in 90 minutes including set up time.

If destination vectors or entry vectors need to be created then the methods are more complex.

Creating a new Entry vector involves amplifying the product with oligos that contain flanking attB sites, then performing a BP clonase reaction (similar to the LR clonase reaction above).

Creating a destination vector involves inserting a *ccdB* resistant cassette with flanking attR sites into the plasmid of interest. This requires transforming specific *E. coli* strains resistant to the toxicity of *ccdB*, which can be purchased or made using a standard competent cell protocol.

Gibson assembly cloning

Gibson assembly works on the principle of using homology to assemble fragments (Gibson, 2011). It uses a combination of an exonuclease, a polymerase, and a ligase in an *in vitro* reaction to assemble multi-fragment constructs without using restriction endonucleases. It is feasible to insert assemblies of 6-10 different fragments into a vector backbone using this approach. The advantages include that 1) no restriction enzymes are required, 2) it is relatively inexpensive, 3) it does not introduce a site or scar like Gateway cloning, 4) it can be used to assembly multiple fragments simultaneously, and 5) it requires fewer steps than traditional cloning. Gibson assembly can be very efficient, but in my experience it is trickier than Golden Gate style cloning. Furthermore, plasmids cannot easily be used as starting reagents, only linear DNA fragments. This is a preferred option when Golden Gate cloning is not an option.

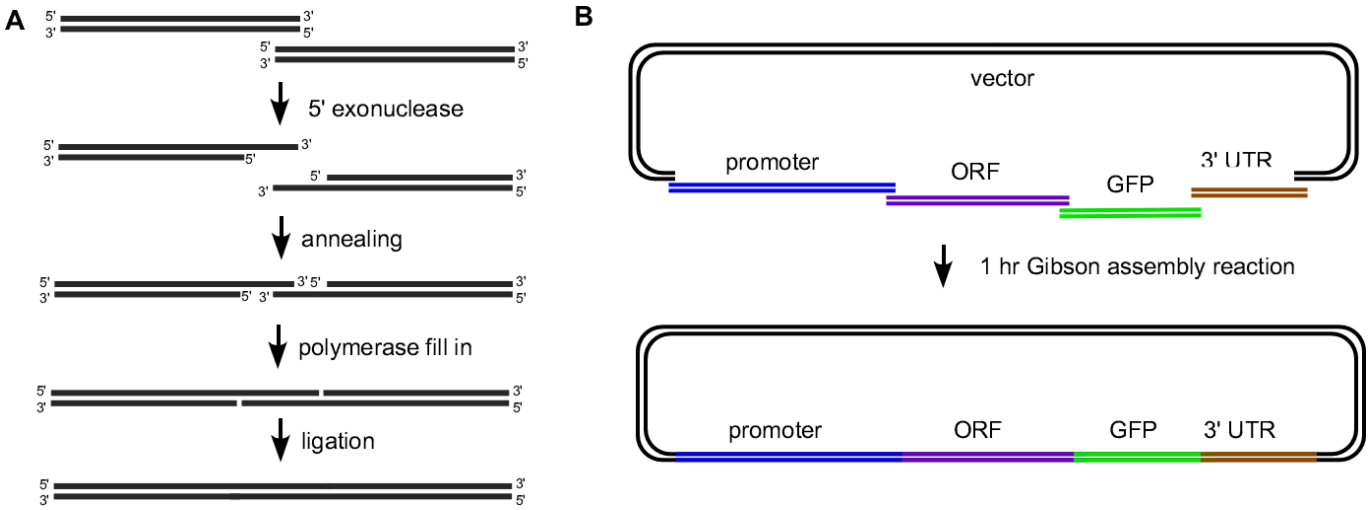


Figure 7. Gibson assembly cloning. A) Fragments are incubated in exonuclease, polymerase and ligase, whose coordinate action leads to fragment ligation. B) Multiple fragments can be introduced simultaneously using this approach at high efficiency. Homology dictates the assembly, thus guiding the formation of correctly structured clones.

Typical procedure for Gibson assembly

In Gibson assembly, most, if not all, of the fragments being inserted are amplified by PCR since homology ends are required for the reaction. The vector source can be PCR amplified, or restriction digested. The key to getting this protocol to work efficiently in our experience is making sure that background of uncut, or single cut vector is minimal. Reactions are quick and simple to perform, but if multi-product assemblies are being produced, multiple insert PCR products need to be created and purified at reasonably high concentration. This can occasionally be challenging.

Site directed mutagenesis

Site directed mutagenesis uses oligonucleotides to make small modifications in a plasmid clone. In the simplest incarnation these simply incorporate changes introduced in via an oligonucleotide into the clone using PCR. This technique can be used to create point mutants, small additions or deletions of sequences and are primarily limited by the length of oligonucleotides that can be commercially synthesized. PCR overlap as a mechanism for introducing changes

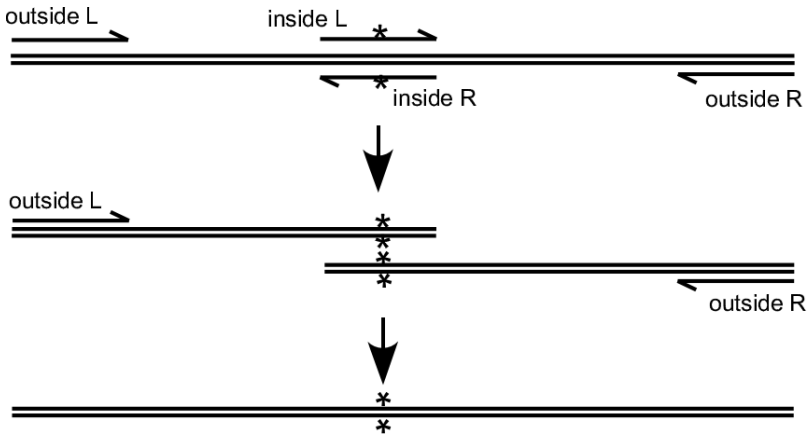


Figure 8. Example using overlap PCR with oligonucleotides containing lesions to incorporate changes in a PCR fragment.

PCR overlap approach

In this method a fragment of a clone is replaced by a PCR fragment that incorporates the lesion using four oligonucleotides (Fig. 8). Two that contain the lesion and two outside primers. The two fragments are amplified separately, then mixed and re-amplified with only the outside oligonucleotides. This fragment is then reintroduced into the parental clone using traditional cloning methods. This approach is often used when the plasmid being mutagenized is large and has appropriate unique sites flanking the region being mutagenized. By avoiding amplification of the whole plasmid, one can be more confident one has not introduced lesions into the rest of the construct.

DpnI mediated site directed mutagenesis

This approaches mutagenizes the plasmid by making a PCR-based copy, then destroys the parental plasmid using the restriction enzyme DpnI. This is based of the fact that DNA from *E. coli dam*$^{+}$ strain (virtually all *E. coli* strains are *dam*$^{+}$) have GATC sequences methylated at A on both strains, and DpnI will only cut methylated GA^{m}TC. Since PCRed DNA is not methylated, this selective cleaves the parental plasmid DNA.

Double primer method

This protocol uses two oligonucleotides, one containing the desired mutation (Fig. 9 & 10). One can add or replace multiple different bases or even delete kb sized fragments. Additions are limited by the size of the oligonucleotides. After the amplification reaction, the DNA is ligated, DpnI digested, and transformed back into *E. coli.* This protocol works exceedingly well for smaller plasmids, and can be successfully performed on plasmids even as large as 15 kb. However, to be absolutely certain other lesions haven't been introduced during the amplification, one needs to re-sequence the whole plasmid. Other derivatives of this approach uses two oligonucleotides that overlap and both contain the mutation (Fisher and Pei, 1997). In this case, no ligase is used. Rather the final product is linear but contains a large duplicated end, which are repaired by recombination after transformation. While this approach originally was considered a linear amplification, rather than PCR, recent experiments suggest otherwise (Xia et al., 2015).

Megaprimer method to lesion multiple sites

The megaprimer method uses two primers each containing a lesion to amplify a large PCR product containing the lesions (Tseng et al., 2008). This product is then purified and used as the primer for the DpnI mediated oligo protocol above. This works reasonably well.

Single primer method for multiple lesions

This approach uses one oligonculeotide per mutation site and can mutate more than one site at a time. While this protocol is less reliable than the megaprimer protocol (only ~25% of colonies will contain the desired mutations), if one is performing multiple mutations on the same piece of DNA than this can save significant time. In this protocol there is an initial kinase (phosphorylation) reaction to allow the primers to be available for

ligation. The polymerase will extend from one primer to another and then a thermostable Taq ligase is used to seal the nicks. This allows multiple mutations to be introduced at the same time.

Typical procedure for site directed mutagenesis

These protocols vary significantly depending on the approach. For PCR based overlap mutagenesis, two PCR reactions, are performed, then the purified products are mixed, and re-amplified with only outside oligonucleotides. Following, that, the procedure is identical to a standard restriction enzyme subcloning protocol.

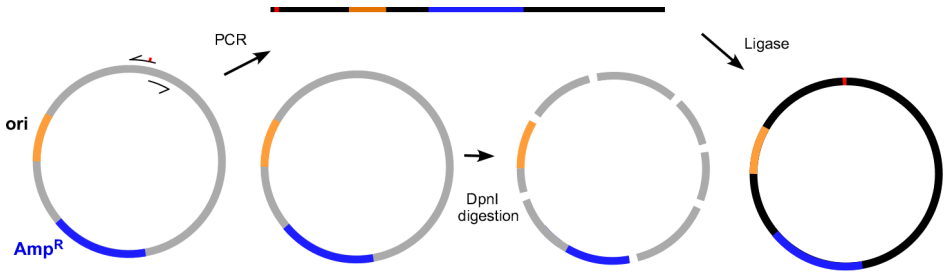


Fig. 9. Example of two primer DpnI assisted PCR based mutagenesis.

DpnI mediated mutagenesis involves a long PCR reaction to amplify the whole plasmid being mutagenized. For large plasmids this PCR reaction can take many many hours, and optimizing the reaction may be required in some cases. The parental unmodified vector is destroyed by a DpnI digestion, followed by transformation. In the megaprimer reactions, a PCR product is first purified, and this serves as the “primer” for a standard DpnI-mediated reaction.

The single primer reaction involves a series of step that first kinase the oligos, followed by a complex PCR reaction that contains both a high temperature ligase and a polymerase. The term single oligo reaction is a misnomer, since the are reverse primers (required for PCR to work), but they come from common vector regions and thus the same primer can be used in every reaction.

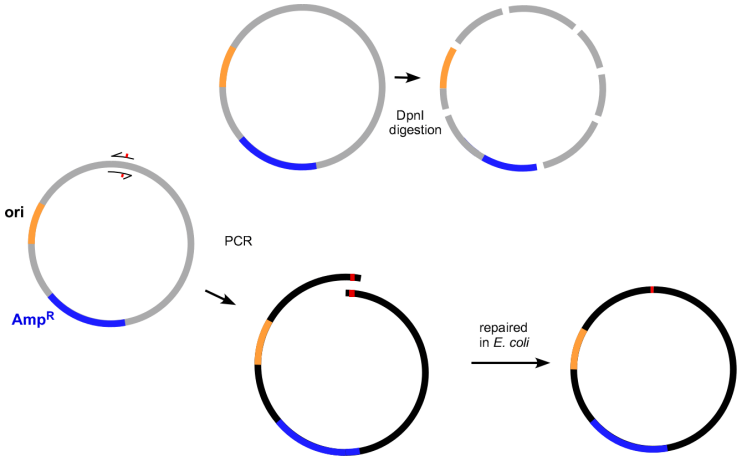


Fig 10. Alternate two oligo mutagenesis method. In this case the oligonucleotides are designed to overlap, so we get a linear amplification. Parental plasmid DNA is cleaved by DpnI digestion. No ligase is used and the nicks are repaired after transformation into *E. coli.*

Detailed methodology for all five methods

In the following sections I provide a detailed protocol for performing all five types of cloning methods outlined above. The approaches I describe are not the only viable methodology of performing these types of protocols. I describe the specific methods that we have found to be most reliable.

Restriction enzyme cloning

In this traditional approach, a vector is digested with enzymes and an insert is digested with the same enzymes or other enzymes that have compatible overhangs. The DNAs are purified, then ligated, then finally transformed into *E. coli*.

For vector DNA one typically digests the vector with restriction enzymes rather than using PCR to amplify the vector sequences of interest, though either is possible in many cases. The reasons why in many cases one digests the plasmid rather than using a PCR to create the DNA fragment are two fold. First, in many cases the vector is large and PCR amplification of large fragments can be challenging. Second, and more importantly, PCR introduces errors at vastly higher rates (even using the most high fidelity polymerase) than replication in *E. coli*. If one wants to be certain one hasn't introduced errors into key elements of the vector that are not selectable in *E. coli* (anything but the origin and drug selection marker), one needs to sequence these after performing PCR!

Insert DNA is much more often created using PCR for several reasons. First, insert DNA is often not available in clone form, and thus in many cases must be PCR amplified from a natural source. Second, insert DNA is much less likely to have restriction sites at suitable positions. Indeed, vectors are often designed to have restriction sites at ideal spots. Third, they often smaller. Fourth, one is occasionally introducing modifications of an insert such as adding an epitope tag.

Transformation of DNA into *E. coli* is the limiting step of this protocol and the characteristics of the DNA transformation method influence the choice in our design of cloning experiments. *E. coli* transformation is effected by the structure of input DNA. Specifically, supercoiled DNA transforms more efficiently than closed circles, and much more efficiently than linear DNA. Thus, it is critical that vector DNA is digested to completion, and that insert DNA also be devoid of supercoiled DNA. Even 0.1% remaining supercoiled parental vector or insert DNA can ruin an experiment.

Two distinct types of transformation methods are commonly used for *E. coli.* The chemical method uses cells incubated in various divalent cations to make *E. coli* competent. This is the method we use almost exclusively. The second method involves resuspending *E. coli* in very low salt buffer, and using electric pulses to transform DNA. We avoid this method because of the limitations that the requirement for a low salt solution place on the cloning methods.

Designing the cloning strategy

Determining which vector to use

In many cases several different options will be available and they may have different restriction sites that are potentially usable. Check the insert sequence to see which are compatible (the site to be used should be absent from the insert, and ideally the site should appear at the junction being created). If at all possible design a strategy that uses two different restriction enzymes that create distinct overhangs to force the orientation and prevent religation of the digested vector. Remember that in many cases what is vector and insert is somewhat arbitrary. For example, if you have a GFP vector and a promoter vector, one can insert GFP into the promoter vector, or the promoter into the GFP vector. One direction may work better than the other depending on how many different sources exist for each component.

Another consideration should be what additional experiments you might perform with the construct. For example, if you intend to integrate the DNA into the genome, it may be wise to insert the DNA directly into the integration vector.

Designing the insert fragment

In the simplest cases, the insert will be nicely flanked by enzymes that match cloning sites in the vector (usually because, the insert was cloned into a vector with a MCS, and a similar MCS is present in the vector in which you would like to introduce the insert). However, in most cases this will not be the case, and PCR will be used to create an insert with compatible ends. Consider whether you are likely to use the insert many times and place it in many different vectors. If that is the case, it may be worth taking the the time to clone the insert into a vector with an appropriate MCS and then use that clone to create the desired clone. If a long range plan for creating many clones is adopted, cloning an insert into a standard MCS vector, and creating vectors with the same MCS can provide significant versatility in creating clones.

Designing primers

If the insert must be amplified by PCR, you will need to design oligonucleotide primers. See the designing primers for PCR section for details on this subject. After designing the oligonucleotides, create the clone *in silico* to make sure you have frame correct, that you haven't introduced a restriction site unintentionally, and that you ordered the proper strand and proper complement for each primer. Double checking here will save lots of time in the long run.

Vector preparation

If vector DNA has not been previously isolated, it must be prepared. If only DNA is available, the vector must be transformed in to *E. coli*, then purified from *E. coli*. For >95% of all work we do, we use standard Qiagen mini-prep DNA. It is quick to isolate and of more than sufficient quality for cloning. Occasionally, we will perform a Qiagen midi-prep to isolate 100-250 µg quantities of DNA. This DNA can be of higher quality if the prep is done correctly. But it is significantly more work.

Insert preparation

If the insert can be isolated by restriction digestion, then one can simply prepare the insert similarly to the vector. However, in most cases, one will be using PCR to amplify the insert DNA. The source of template can be a complicated decision. Sources of template for *C. elegans* work include *C. elegans* wild type genomic DNA (~50 ng). Alternatively, if cDNA is being sought, a first strand cDNA synthesis can be used. Lastly, a plasmid source can be used, if a clone of the insert is available. While a plasmid template is the easiest to amplify from, it introduces the additional complication of potential contamination with template. If possible, choose a plasmid with a different antibiotic selection marker which avoids the problem. If plasmid is the template, even 1 ng is more than sufficient. Because, grabbing 1 ng often requires diluting a plasmid prep, often much more is used. While this won't harm the PCR, it does increase potential for contamination. When plasmid template is used it must be removed or destroyed. This is usually done using the restriction enzyme DpnI which digests only *dam*$^{+}$ G^{m}ATC methylated DNA.

Detailed protocol

Step 1: Design oligonucleotides to amplify the product of interest

Design a pair of oligonucleotides primers with appropriate restrictions sites at the 5' end of each primer. See design of oligonucleotide primers for PCR for an extended discussion of this issue.

Step 2: PCR amplification of insert DNA

See the PCR section for a discussion of PCR methods

Reaction (set up on ice[11]) total 25 µl

16-x µl	H_20 (cold)
5 µl	5 X Q5 polymerase buffer
2 µl	dNTPs 2.5 mM
x µl	template DNA (see above)
1/2 µl	25 µM primer A (55°C T_m)
1/2 µl	25 µM primer B (55°C T_m)
1/4 µl	Q5 polymerase

Amplify 98°C for 0:30 + 35 cycles (98°C for 0:10; 58°C for 0:30; 72°C for 1:00/kb of insert size)[12]

Optional- for plasmid templates- add 1/2 µl DpnI, incubate 15 minutes at 37°C.
This only needs to be done if the selectable marker on the insert plasmid template is the same as the vector.

Optional- run out 1µl on 0.8 % TAE agarose gel to see if reaction worked. If not, see **Troubleshooting PCR**.

This PCR reaction can be kept at -20°C for many days.

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[11] We start PCR reactions on ice, then transfer them to the PCR machine after starting the machine and it has gone above 72°C.

[12] These PCR cycling conditions are specific to Q5 polymerase with 55°C T_m, and a ~ 1kb with an expected 1 kb PCR product. Different high temperature polymerases will have very different conditions. For example, PrimeStar polymerase required a very short annealing time, and Taq polymerase is less stable and will not tolerate 98°C denaturing.

Step 3: Clean-up purification of PCR product

Add 150 µl 1:9 PBI[13] to PCR reaction (5-6 volumes of PBI).

Load onto Qiagen or Monarch mini elution column.

Spin 30 seconds in microfuge at full speed to bind to column. Remove binding liquid.

Add 300-500 µl PE buffer, spin 30 sec. Remove PE.

Add 300 µl PE buffer, spin 30 sec. Rotate tube 180 degree, Spin 1 minute.

Transfer column to new 1.5 ml eppendorf tube.

Add 10 µl of TE. Spin in microfuges at lowest speed setting for 1 minute to drive TE into column.

Incubate 1 minute, then spin at full speed 1 minute to elute from column.

optional - to increase yield, but lower concentration, add another 10 µl TE and repeat elution.

optional - heating the column during the elution, by placing column in a 65°C bath before spinning at high speed to elute DNA can help yield with large DNA fragments (>3 kb).

Determine concentration of DNA using a NanoDrop spectrophotometer. Yield for such PCR reactions should be on the order of 1-2 µg (50-100 ng/µl product).

Step 4: Digestion of products

See the restriction digestion section for a detailed discussion of this topic

Vector digestion (order of addition)	Insert Digestion
(11.5-X) µl of H20	(11.5-X) µl of H20
1.5 µl 10X Buffer	1.5 µl 10X Buffer
X µl vector (~200 ng)	X µl of insert PCR product (~200 ng)
0.5 µl enzyme A	0.5 µl enzyme A
0.5 µl enzyme B	0.5 µl enzyme B
Incubate 1hr at 37°C	Incubate 1hr at 37°C

In most cases, a 1 hr digestion is more than sufficient to digest DNA, but in unusual cases more extended digestions may be needed. See the restriction digestion section if digestion of DNA is problematic.

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[13] PB, a mixture of 5M guanidine HCl and 30 % ethanol, is a typical binding buffer used for most silica-based purification column. The "I" is an indicator dye that turns from yellow to purple above pH 7.5. DNA will not bind to the column if the pH is above 7.5. We use diluted PBI because, small ssDNAs (primers) and dsDNA (primer dimers) bind to the column less tightly than larger DNAs, and in diluted PBI, small DNA are not purified. Using 5 volumes of PBI + 1X Q5 PCR reaction buffer, we found using PBI > 33 bp dsDNA binds fully
1:1 (PBI: H20) > 46 bp dsDNA binds fully, 33 bp dsDNA binds partially
1:4 (PBI: H20) > 64 bp dsDNA binds fully, 46 bp dsDNA binds partially, < 33 bp dsDNA does not bind
1:6 (PBI: H20) > 88 bp dsDNA binds fully, 64 bp dsDNA binds partially, < 46 bp dsDNA does not bind
1:9 (PBI: H20) > 200 bp dsDNA binds fully 148 bp dsDNA binds partially, < 100 bp dsDNA does not bind

PBI is diluted with water with the addition of 5 µl of 3M KAc pH 6.0 per ml
Note that there is some sequence specificity to these cutoffs that we do not completely understand.

Step 5: Gel purification of products

Pour 0.8% low melt agarose[14] TAE[15] gel.

Load 10 µl of vector and insert digests on gel in different wells. Run gel ~ 20-30 minutes until bands are well separated. The DNA is visualized using ethidium bromide[16] on a trans-illuminator with 300 nM UV bulbs. Use 100 μl capillaries to "punch out" vector and insert band and place in tube (Figure 11). Do not take the gel off of the slide[17]. Photograph gel. These punches can be kept in the refrigerator for weeks

Step 6: Ligation

Heat gel punches to 65°C for 3 minutes.

Set up ligation

<u>Ligation mix (in order of addition)</u>

14 µl H20

2 µl 10X ligase buffer

2 µl vector punch [18]

2 µl insert punch

1/4 µl T4 DNA Ligase

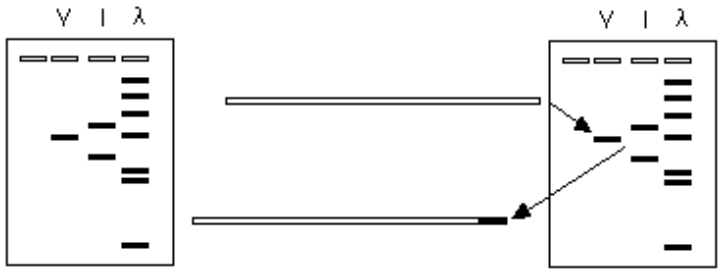


Figure 11. Purify band by collecting DNA in a capillary.

Incubate at 15 C for 1 hr to overnight. 1 hr is typical, but o.n. will increase efficiently somewhat. Even 5-10 minutes is sufficient for most double sticky-end cloning procedures. Blunt end ligations are 20 fold less efficient and o.n. will often help these. This ligation can be frozen at -20°C for later transformation.

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[14] We pour gels on slides 50 mm x 75 mm using 10-12 ml of agarose. Surface tension holds the liquid on the plate. Use the lowest concentration of agarose that is appropriate. If one is separating two similar sized ~ 400 bp bands, one may need 1.5% or 2.0%. If one is purifying a very large plasmid insert, 0.5% agarose may be sufficient. Note that low melt agarose gels are fragile. We pour them in the cold room. Pull combs out gently. We use exclusively SeaPlaque low melt agarose. It is expensive, but ligations work well in this agarose. If you accidentally pour a regular gel and the fragment won't melt, you can freeze the tube with the punch -20°C or briefly at -80°C or liquid N2, thaw, spin at high speed in a microfuge for 5 minutes, then pull a few µl of the liquid from the top. Freezing breaks down the agarose, and releases some of the DNA. This is much less efficient than a low melt agarose, but can save the day.

[15] Do not use TBE (Tris Borate buffer). While it works better to run the gels, it is not compatible with ligation.

[16] To visualize the DNA , we use ethidium bromide (.05 µg/ml) in the gel and buffer. Ethidium bromide does not interfere with the ligation or transformation. Although ethidium bromide is often thought to be dangerous, it is actually not very mutagenic or toxic. See http://blogs.sciencemag.org/pipeline/archives/2016/04/18/the-myth-of-ethidium-bromide

[17] Direct visualization of DNA in a gel without a glass layer to shield from UV will very quickly damage DNA to the point where it will not clone. If you wish to see the DNA through the glass slide efficiently using a standard trans-illuminator use low iron glass slides. These look clear in cross section. Regular glass looks green in cross-section. UV transparent plexiglas is also available and works fine.

[18] In theory, one would like to have about 1:1 molar ratio of insert to vector. But, this is highly flexible. My general experience is that if you can see the DNA on the gel ,with the 10 μl punch you have sufficient material to perform the cloning. If vector is very bright, or insert is very small adjusting these volumes of DNA addition (without increase fraction of agarose > 30% reaction) can improve efficiency.

Step 7: Transformation[19] of *E. coli* DH5α

Thaw competent cells on ice (takes ~ 10 minutes).

Heat ligation to 65°C for 3 minutes to melt.

Meanwhile, aliquot 100 µl of competent cells into pre-cooled 1.5 ml Eppendorf tubes.

Add 1-2 µl of ligation mix, quickly vortex and return to ice.

Incubate 30 minutes on ice.

Heat shock 40 sec at 42°C and return to ice

Incubate 5-10 minutes on ice.

Add 700 µl of SOC media.

Incubate 1 hr at 37°C while shaking.

Plate 100 µl on agar plate with appropriate selection marker. Incubate overnight.

The next morning one will have a plate with 0 to >10,000 colonies on it. Hopefully, between 100 and 1000 if one has done a good job performing the procedure.

Assessing ligation results

How many colonies you get depends on only two factors. How competent the cells are and how well your ligation worked. However, more colonies does not necessarily mean the experiment went well. In an experiment where one is ligating an insert with a vector, and the vector or insert did not get digested to completion, uncut vector or insert plasmid both will transform with very high efficiency and result in many colonies, but no correct colonies. On the other hand, if your cells are not very competent, you may get no colonies even if your *in vitro* ligation worked very efficiently.

Controls for assessing experimental success

Typically two types of controls are performed to assess if an experimental ligation has worked well. An experienced cloner often does not perform these controls as they use substantial reagents and time to perform and offer very little benefit if the experiments are working well. But, if we are having troubles, we quickly revert to using them to identify the source of a problem.

<u>Experiment:</u>

vector + insert + ligase

<u>Control 1 (most useful):</u>

Vector + insert (NO ligase)

Getting colonies on this control indicates a contamination of uncut plasmid in either the vector or the insert. The typical sources of this contamination are poor digestion of the vector or insert or an external source of contamination of plasmid DNA (e.g. someone went into the ligase with a DNA contaminated pipette tip).

<u>Control 2 (often useful):</u>

Vector + ligase (NO insert)

The usefulness of this control depends on the structure of the cut vector. If the vector is double cut and

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[19] There are two general types of transformation procedures. We use chemically competent cells which are made by incubation with a divalent cation salt solutions. See preparing competent cells for details. Another type of procedure involves electroporation of DNA into E. coli. These cells are prepared by incubating cells in low salt solutions (usually water). DMSO or glycerol is also included in the last buffer for frozen cells. Frozen cells work equally well as fresh cells.

the ends are not compatible this control often yields substantially fewer colonies than vector + insert[20]. If the cut ends of the vector are compatible (e.g. single cut vector), then one expects many colonies, and should yield many more colonies than control 1.

Control 3 (potentially useful)

Insert + ligase (NO vector)

If an insert is contaminated with linear DNA that carries an origin of replication and a drug resistance marker or supercoiled uncut DNA one could get insert contamination. This control tests for this source of contamination.

Troubleshooting experiments that yield many incorrect clones

While the controls above will of point to the source of the problem, they often don't offer a solution. Defining what the molecular structure of contaminating colonies will often point to the source of contamination. For example if you mini-prep 12 colonies, if they all look like the parental vector this would suggest the parental vector either was not digested or re-assembled during the ligation. On the other hand, if the colonies contain DNA that looks like the parental insert clone, it would suggest the insert was not digested to completion. If they look like neither, it would suggest an external source of contamination.

The above controls assess the quality of the input reagents and the efficiency of the ligase reaction. However, under certain circumstances the source of contamination has noting to do with the ligation and DNA inserts, but is another source of contamination.

Testing for contamination

Transforming competent cells with no DNA added will test for contamination of the competent cells themselves, or the SOC growth media. Formally, colonies on a no DNA added control can result from contamination of pipetters or pipette tips.

Water, ligation buffer, and ligase can also be transformed to identify a source of contamination, though usually just tossing all these reagents and using fresh ones is a faster approach (unless such contamination is occurring repeatedly, and the methodology driving contamination needs to be defined).

Troubleshooting failure to get any colonies

Apart from foolish errors like plating the ligation on the incorrect antibiotic selection plate, the usually reason for failure to get any colonies usually results from using poor competent cells.

Competency of competent cells

Test the competency of the the competent cells by transforming with 10-100 pg of supercoiled DNA. If you purchase competent cells, they often come with control DNA to test competency. For lab made competent cells (which I highly recommend doing), one can simply use a standard lab vector. However, keep in mind that competency is also effected by size of the plasmid [21]. You will get a substantially higher competency if you use a small plasmid for your assay.

Testing ligase

Another possibility is spoiled ligation buffer. It contains ATP and that can hydrolyze. Replace the ligation buffer. It is also possible the ligase itself is dead. One can test if ligase is working by digesting a plasmid that cuts several times with a "sticky end" enzyme, purifying the DNA, then ligating 100 ng in a 10 μl

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[20] With double cut vectors, it is possible to have the ligation work well and still have more colonies on the vector, no insert + ligase control, than on the experimental ligation. This is because colonies that appear on his no insert control usually represent single cut vector re-ligated (one of the two enzymes did not cut to completion). In the experimental setting these single cut DNAs are often ligated to insert, and yield linear products with non-compatible ends (and these do not yield colonies). So effectively, background is lowered in the experimental condition.

[21] First, the larger the plasmid, the less molecules per mass. Secondly, larger DNA transforms less efficiently into most *E. coli* strains.

ligase reaction for 5 minutes, and run the reaction out on a gel. Even a 1:50 dilution of ligase should ligate the bands together into a smear at the top of the gel.

Damage of DNA during purification

Another common issue is damaging the DNA during gel purification. One must keep glass between the gel and the UV light. We use low iron glass slides, which block very little of the longer wavelength and still allows us to see the DNA without low wavelength UV damaging the DNA.

Poor quality input DNA

A third common problem is poor quality of the input DNA due to salt or ethanol contamination. T4 DNA ligase and transformation are both inhibited by high salt. Forgetting the PE wash in the mini prep, or not spinning hard to remove all the PE buffer before eluting the DNA are common mistakes that cause really poor quality DNA.

Skipping the PCR purification

While it may be tempting to skip the PCR purification if the reaction yields are high, do not do this. The PCR purification is critical to perform because residual polymerase activity can re-fill or chew back overhangs after DNA digestion.

Identification of correct clones

The three basic options for identifying proper clones are DNA restriction digestion, PCR, and sequencing. In my opinion, PCR is the least appropriate method, though it is widely used by many. Why do I think it is a poor method. Three reasons:

1) PCR is highly susceptible to contamination. A 1:100,000 contamination of new clone with a prior clone that contains the same fragment new clone will yield PCR product. Contamination can come from many sources including pipetters, buffers, water source or even (often) restriction enzymes. Lastly, in the specific case for my lab, the fact that we often re-use columns for plasmid purification and insert isolation. In our lab contamination is rare : only 1 clear case of contamination has been documented in my lab. Oligonucleotide stocks were contaminated with a DNA template. We realized the problem quickly because we were genotyping animals from a cross genotype ratios we extremely skewed. I would guess that we have incorrect clones in our collection from our prior use of PCR for plasmid identification.
2) The second reason is that PCR is not comprehensive. PCR can document the existence of a fragment of known size with known ends, but it does not interrogate the whole plasmid DNA molecule.
3) PCR is not time efficient. If cloning methods are working well, and one does a reasonable job in cloning design, most colonies you isolate from cloning experiments will be correct. Certainly, for virtually all experiments, greater than 1 of 6 colonies will be correct. Setting up a PCR reaction, running the reaction, then running a gel to analyze it only tells you this colony might be correct. But one still has to grow up the colony to isolate the DNA. So one spends a minimum of 2 hrs to identify the colony one should grow up.

Our approach is just go directly to plasmid DNA preps. They are quick to perform, and they need to be performed on the correct clone regardless. Since I re-use my mini prep columns, there is little cost associated with this. In addition, I argue this takes less time and effort compared to performing PCR, then subsequently performing mini preps on a smaller set of clones.

How many colonies I prep depends on how many clones I am isolating, how hurried I am to get it, and how well I feel the experiment went (depending on the quality of the products I amplified, the enzymes I used, history of performing similar cloning etc). For example, if I am cloning a new sgRNA template into DR274 U6, 3 colonies will give me 3 successful clones 90% of the time. By contrast, if I am doing a 6 insert SapTRAP clone, and the PCR arm products yields were sub-optimal, I might mini-prep 12 colonies.

I inoculate test tubes containing 3 ml of LB[22] with a colony using a pipette tip. 1.5 ml of the culture is prepped up using the our standard mini prep protocol.

Analysis of clones

As I argued above, PCR is a poor method for confirming clone identity. Small levels of contamination can easily fool you. Instead, to determine whether an isolated clone DNA encoded the correct plasmid, I perform restriction digestions. Typically one wishes to digest the DNA with a restriction enzyme that will give a unique pattern for the clone of interest that distinguished it from the parental plasmids (vector and perhaps insert plasmid if the insert comes from a previously made plasmid). It is typically a bad idea to use the enzymes used to create the clone to analyze the clones. While it will confirm the correct sized insert is in the vector, it does not confirm the identity, and since one is often selecting for the size of the insert by purifying a band of a select size, one would expect almost every clone to have that sized insert. The better approach is to use and enzyme the cuts the vector and insert a few times (2-5 in total) that will produce a distinct pattern of non-overlapping bands. I choose enzymes that work robustly whenever possible. These include BamHI, BglII, DraI, EcoRI, EcoRV, HindIII, KpnI, NcoI, NheI, PstI, PvuI, PvuII, SacI, SacII, SalI, SphI, SmaI, XbaI, XhoI. Many others are suitable, but the enzymes listed above are highly reliable, not prone to unusual problems associated with restriction digest analysis, and relatively inexpensive.

In certain circumstances sequencing is the best way, and occasionally the only way, to determine if you have the correct clone. For example if you have created a specific mutant version of a gene and the lesion does not alter a restriction site.

The only times I use PCR to characterize a clone is if I am having a serious difficult identifying the correct clone among the colonies and I have reason to believe repeating the experiment will not improve my ligation quality. However this is very rare. In these cases, I often pool colonies by touching each colony with a sterile toothpick, streaking the colony on a plate, then placing the toothpick in a tube with 100 µl of water, and spinning the toothpick to release some *E. coli* in the tube. I repeat this for 12 colonies, then use 1 µl of the water *E. coli* mix as the template for PCR to test if any of the dozen colonies have the product I am looking for. If I get a positive, I mini-prep each of the dozen colonies to isolate my clone. If this approach doesn't yield a positive in testing 72 colonies using 6 PCR reactions, something is very wrong with the cloning experiment.

Alternative Approaches

Hybridized oligonulceotides as inserts

A pair of complementary oligonucleotides with appropriate overlaps can be utilized as an insert. Although the 5' ends are not phosphorylated, a single bond on each end of the insert will still be formed during the ligation, and the double nicked resulting circular molecule will be repaired *in vivo.*

Mix 1 µl of 100uM stock of each oligo in 100 µl of total volume of water or annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 0.1 mM EDTA). Heat to 95 for 5 minutes and slow cool (- 5 to 10 °C /minute). We typically use 1 µl of this mix (~ 40 ng of a 60 bp oligo pair) with ~ 50-100 ng of vector in a 10 µl ligation. That is 1 pmol of insert for ~ 25- 50 fmol of vector. This is a high insert to vector ratio, but it works well for us. One can use a gel purified insert for this protocol, but there is no purpose to purifying the hybridized oligonucleotides.

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[22] I typically do not add antibiotics to my growth media to grow up plasmids. I find it does very little to improve yields. The reason is that ampicillin does a horrible job of selecting for the plasmid containing cells in liquid culture. Carbenicillin, kanamycin and tetracycline work better in liquid. I find the two things that correlate best with yield are how big the colony is on the plate, 2) that the plates are fresh, and haven't been stored in the cold for long periods of time.

Gel Purification of large amounts of DNA

Occasionally, a PCR or some of DNA sample contains a mixture that can be separated by electrophoresis, but that cannot be synthesized independently from each other. For example, a PCR from genomic might yield both the correct sized product and an alternate product from a homologous locus. If one is performing a reaction which requires large amounts of DNA, purification from a gel may be the only alternative to isolate a pure product. In these rare cases, we run the DNA on a gel, "melt" the gel using Qiagen's QG buffer[23], load it on a small DNA purification column, then elute the DNA.

Load the DNA samples in multiple wells so as to prevent smearing, use a razor blade to cut our the band in as little gel as possible. Keep in mind that you want to do this on glass to prevent DNA damage. Weigh the gel slice to calculate volume (1g/ml). Add 3 volumes of Qiagen QG buffer, heat to 50°C for 10 minutes. This will dissolve the gel (add more QG buffer it it does not), apply in 600 µl aliquots to a Monarch mini-column (5 µg capacity). Wash with 600 µl of QG buffer, wash with 600 µl of PE, and then 300 µl PE, spin dry for 1 minute, and elute in 10-40 µl of TE. Yields are typical 50-80% of input DNA. Qiagen claims that adding 1 gel volume of Isopropanol after the 3 volumes of QG will increase yields for small (<500 bp) or (large >4000 bp) fragments. I have not tried this.

[23] 5.5 M guanidine thiocyanate (GuSCN), 20 mM Tris-HCl, pH 6.6 (25°C).

Golden Gate Cloning

Golden gate cloning is a powerful technique, but requires careful design of oligonucleotides and specialized vectors that contain the type IIS restriction sites being used for the assembly **only** at the designed positions in the insert fragments. We now use the method to create multi-insert plasmids both using the enzyme SapI to create vectors for integration (see separate document on SapTRAP vector assembly) and with BsaI to create multi-insert vectors that feed into the SapTRAP protocol.

The basic methodology involves designing PCR products that contain the fragments of interest to be assembled with the appropriate Type IIS sites on the ends of the PCR fragment, positioned to keep frame (if appropriate) and with 3 or 4 base overhangs that are unique and appropriately complementary to create the desired assembly. If an insert contains the site being used, the site can be "removed" by splitting the fragment into to fragments, which modify the site when assembled together. If multiple sites are in the insert, one option is to have the insert synthesized with the sites lesioned.

If an insert fragment will be used repetitively, one reasonable option is to clone the fragment with the sites into another vector with a different drug resistance marker than the final desired assembly. Then the plasmid, rather than a PCR product can be used in the assembly.With planning, many related constructs can be efficiently create without the need to create new insert DNAs.

Detailed Methodology

Step 1: Design primers to amplify insert fragments

I find the best way to perform this design is to assemble the product you are trying to create *in silico*, then design the oligonucleotides from that template creating the junctions through the addition of the flanking Type IIS sites (usually BsaI or SapI). For a SapI reaction we are typically adding the sequence AAGCTCTTCN/NNN 5' of the homology section of the oligo, and for a BsaI reaction we are adding AAGGTCTCN/NNNN. See designing primers for PCR for a discussion of primer design, and the SapTRAP cloning guide for a detailed example of designing oligos for this methodology.

Note that small fragments (such as those encoding a His6 tag, or a FLAG tag) can also be added as hybridized primer pairs. The hybridized oligonucleotides should create the proper overhangs. If fragments consisting of oligonucleotides are not consecutive fragments in the assembly, they can even be added without phosphorylating the oligonucleotides. The final product will contain unligated nicks that can be repaired by *E. coli*. Some protocols recommend add T4 polynucleotide kinase to the reaction to phosphorylate the oligonucleotides. This is essential if consecutive fragments in the assembly are primer pairs.

Step 2: PCR amplification of insert DNA

Perform as described in PCR amplification of insert DNA in the restriction enzyme cloning section.

Step 3: Clean up purification of each PCR product

Perform as described in Clean-up purification of PCR Products in restriction enzyme cloning section.

Step 4: Ligation

2	µl	6X SAP reaction buffer
x	µl	H_20 (to 12 µl total)
y	µl	vector (50 ng)
$z_1..z_n$	µl	inserts (5 - 25 ng each [24])
1/2	µl	Type IIS enzyme (e.g. BsaI or SapI)
1/4	µl	T4 DNA ligase

[24] The idea is to have approximately a 1:1: ...1_n ratio of the n inserts and a ~ 1/2 molar ratio of vector such that vector is limiting. Very small inserts (<~100 bp can be problematic if added in too high a concentration as they compete with complete cleavage of vector. The ratio becomes more critical as insert number increases.

1/4 µl T4 polynucleotide kinase *(optional)*

Incubate 15 min 37°C, followed by 3 X (5 min at 15°C, 10 min at 37°C), 5 min at 55°C, 5 min 80°C[25].

(optional) - add 1 µl of 10 mM ATPase, 1 µl of Plasmid-Safe nuclease, and incubate 1 hr at 37°C[26] .

Step 5: Transformation of DH5α

Perform as described in Transformation of *E. coli* using 1µl of ligation.

Typically one will get many colonies and >80% should be correct for assemblies with less than 5 inserts. For SapI mediated SapTRAP assemblies (which usually have 6-8 inserts), usually > 50% of the colonies are the correct structure.

Step 6: Analysis of clones by mini prep and restriction digestion

We usually grow up 4 colonies and prepare DNA using a standard mini-prep protocol and confirm the structure of the clone by one or two distinct digests. Sequence confirmation of the clone is recommended. Virtually all clones will be correct if one uses a high fidelity polymerase, and is careful about the restriction digest analysis to confirm the structure of the clone.

Troubleshooting Golden Gate reactions

No colonies

The most likely possibilities are:

- Poor efficiency competent cells.
- Improper design of the assembly such that no circular product can be formed.

Clones are partial assemblies missing a specific fragment

The most likely possibilities are:

- Insert is contaminated with a primer dimer assembly that is used in place of the insert in the reaction
- Improper design of the assembly such that the insert is skipped.

Clones resemble input vector

The most likely possibilities are:

- Type II S enzyme is not performing properly (old enzyme, or not enough added)
- Sample was not well mixed and some vector did not “see” enzyme.
- Vector is in excess and the reaction was not heat inactivated. The ligase religated vector between the end of the last high temperature digestion step, and the transformation.

Clones resemble other plasmid

The most likely possibility is that an insert was prepared from a plasmid template with the same drug selection marker as the final product, and it was not cleaved or removed during insert purification.

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[25] Conditions for effective ligation are varied. For 10 inserts, the Voytas TALEN protocol recommends :
10X (37°C for 5 min, 16°C 10 min), 55°C for 5 min, 80°C for 5 min
NEB recommends:
1 to 4 inserts 37°C for 1hr, followed by 55°C for 5 min
5 to 10 inserts 30X (37°C for 1 min, 16°C 1 min), 55°C for 5 min
11 to 20 inserts 30X (37°C for 5 min, 16°C 5 min), 55°C for 5 min

[26] For TALEN vector assembly this ligation is followed by an incubation with plasmid safe nuclease to prevent linear fragments from reforming circular plasmids after transformation. However, TALEN assembly is a special case where each TALEN module is extremely homologous to the others and partial assemblies can be repaired via homology in *E. coli*.

Gateway Cloning

The Gateway cloning approach requires significant upfront cloning before deriving the benefit of rapid construction of new clones. However, once the component parts have been built, creating working clones is quite simple and usually very reliable. To establish the system, on must create a set of "Entry" vectors, and either create or acquire appropriate "Destination" vectors. With both of those sets of vectors in hands, on can create working plasmids by combining Entry and Destination vectors.

Detailed Methodology

Creating Entry vectors

Entry vectors are created by creating PCR products that contain attB sites on the ends of the product. These sequences are appended to the 5' of the primers being used to amplify the product. After purification, these are mixed with a DONR plasmid in a BP reaction to create an entry clone.

Step 1: Designing Entry vector primers.

The *attB* sites used to recombine into pDONR 221[27] are listed below. If one is creating entry clones for a multi-insert gateway system, then one must make sure one is using the correct set of *att* sequences to create the proper insert ends for a BP reaction into the proper donor vector[28].

attB1 GGGG-ACA-AGT-TTg-tac-aaa-aaa-gca-ggc-tNN

attB2 GGG-GAC-CAC-TTT-GTA-CAA-Gaa-agc-tgg-gtN

Frame is marked in these vectors and should be maintained if fusion are being created. The gene specific portion of the oligonucleotide should have a T_m of >55°C.

Designing entry vectors for the multi-clone gateway system is more complicated. The oligo nucleotides for classical pDONR™ P4-P1R, and pDONR™ P2R-P3 are included in the footnotes. However, significant planning should be performed before initiating an extensive set of multi-insert gateway cloning strategy.

Step 2: PCR the insert fragment

Perform as described in PCR amplification of insert DNA in the restriction enzyme cloning section.

Step 3: Clean up purification of each PCR product

Perform as described in Clean-up purification of PCR Products in restriction enzyme cloning section.

Step 4: Perform the BP reaction

PCR product (~75 ng)	1µl
pDONR221 (~150 ng)	1µl

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[27] Improved and more efficient *att* sites used to recombine into pDONR 221:
attB1.1 GGG-GCA-ACT-TTg-tac-aaa-aaa-gtt-gNN
attB2.1 GG-GGC-AAC-TTT-GTA-CAA-Caa-agt-tgN

[28] The att sites used to recombine into pDONR P4-P1R:
attB4 GGGG-ACA-ACT-TTg-tat-aga-aaa-gtt-gNN
attB1r GGG-GAC-TGC-TTT-TTT-GTA-Caa-act-tgN

The att sites used to recombine into pDONR P2R-P3:
attB2r GGGG-ACA-GCT-TTc-ttg-tac-aaa-gtg-gNN
attB3 GGG-GAC-AAC-TTT-GTA-TAA-Taa-agt-tgN

5x BP reaction buffer	1µl
H_2O	1µl
BP clonase	1µl
	Σ 5µl

Remove BP clonase from -70°C, thaw on ice, vortex briefly, add 1µl to the reaction and return to -70°C.

Incubate reaction mix at 25°C for 1h, (then 4°C forever).

Add 1/2 µl 10 mg/ml proteinase K solution, incubate for 10 minutes at 37°C (then at 4°C forever).

Step 5: Transform into DH5α

Perform as described in Transformation of *E. coli* using 1 µl of reaction, and plate 100 µl of transformation mix on LB + antibiotic and grow at 37°C overnight.

Step 6: Analysis of clones

Select 4 colonies to grow up and isolate DNA using the mini-prep protocol. Analyze the resulting plasmid by restriction digestion. Confirm by sequencing if an exact sequence is essential.

Creating a destination vector

To create a destination vector one needs to insert the *attR1/2* sites in the proper position in the vector of interest. The *attR1/2 ccdB* Cm^R cassettes come in all 3 reading frames (Figure 12) . The cassette must be either amplified or purified by digestion from from appropriate Gateway cassette clone[29], and then inserted into the vector using restriction enzyme cloning or Gibson assembly cloning. Remember that the ligation or Gibson assembly reaction must be transformed into a *ccdB* resistant strain. Note that the Destination vector cannot have the same antibiotic resistance as the Entry vector. Since pDONR221, which is used to create entry vectors is kan^R, this means using either Amp^R or tet^R resistant vectors. A set of Zeocin resistant pDONR vector are also available.

[29] NM1343 pBS RfAf, NM1344 pBS RfBf, NM1356 pBS RfC.1f

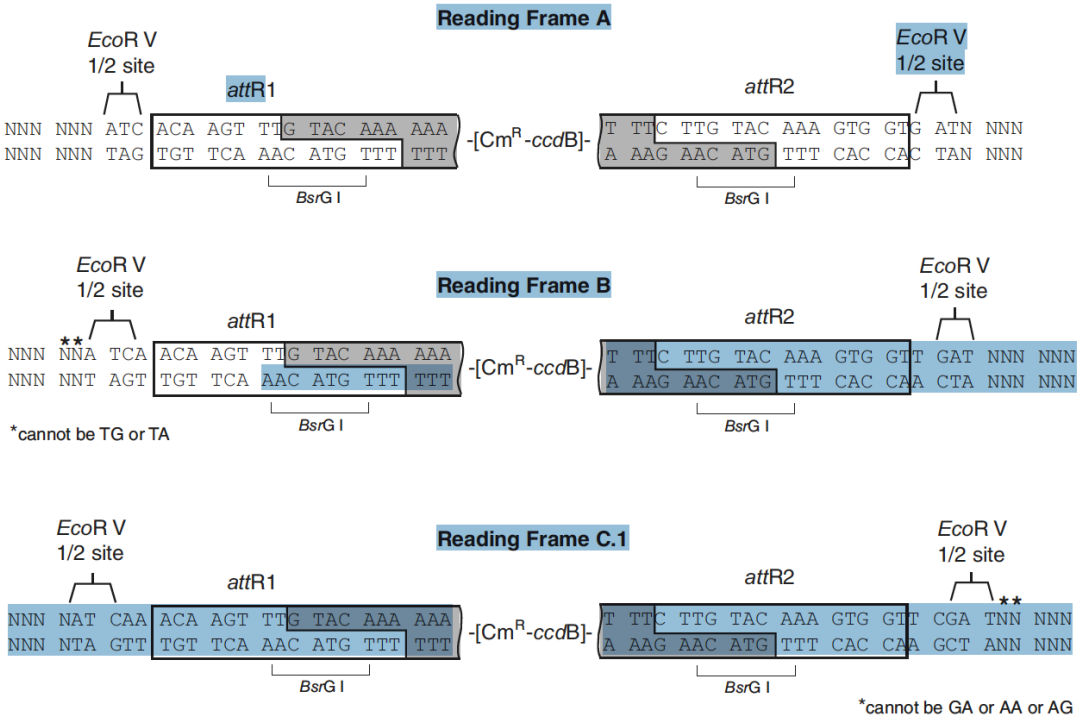


Figure 12. Reading frame of the 3 attR cassettes from Invitrogen.

Using the LR reaction to insert Entry clones into Destination clones

Final product vectors are created by a recombination event between a Entry vector and a Destination vector using an LR reaction.

Step 1: LR reaction

pDEST vector (50-100 ng)	1µl
pEntry vector (50-100 ng)	1µl
5x LR reaction buffer	1µl
H2O	1µl
LR clonase	1µl

Remove LR clonase from -70°C, thaw on ice, vortex briefly, add 1µl to the reaction and return to –70°C.

Incubate reaction mix at 25°C for 1h, (then at 4°C forever).

Add 1/2 µl proteinase K (10 mg/ml), incubate for 10 minutes at 37°C, (then at 4°C forever).

Step 2: Transformation into DH5α

Perform as described in Transformation of *E. coli* using 1µl, and plate 100 µl of the transformation on LB + the appropriate drug selection marker. Grow at 37°C overnight.

Step 3: Analysis of clones

Select 4 colonies to grow up and isolate DNA using a mini-prep protocol. Analyze the resulting plasmid by restriction digestion. Sequencing of the resulting clone is not needed if the parental Entry and Destination vectors have previously been characterized by sequencing.

Gibson assembly cloning

Gibson assembly cloning uses homology to direct the assembly of multiple fragments *in vitro*. Linear PCR fragments (or formally any purified linear piece of DNA and linear vector are mixed with the Gibson assembly mix (or Hi-FI mix), and then transformed into *E. coli*. Gibson-type assembly works best with 20-25 bp overlaps if one is using the commercial NEB Hi-Fi assembly mix (what we use). One common difficulty of this approach is getting all of the component DNA fragments concentrated enough to assemble the reaction.

Detailed methodology

Step 1: Designing the construct assembly

Choice of Vector

The key to Gibson-style assembly working is low background of vector re-ligation. Double digested vectors work well for this. It is very important that the digestion go to completion. There is no need to gel purify vector. As an option, one can use a vector with counter-selection such as a vector with a *ccdB/Cm*^{R} cassette inserted in the middle of the polylinker, such that uncut vector is selected against. About 50 ng of vector are needed per reaction.

Designing insert primers

Designing of the PCR primers can be aided by on line assembly tools such as those available at NEB's website. I design the oligonucleotide manually by building the construct I am trying to build *in silico* with clear demarcations of the distinct fragments I am placing in the clone. I then design oligonucleotides that overlap by 25 bp from this *in silico* template. I use 58°C annealing temperature for the homology section of the oligo and grab the 25 bases 5' of the homology section being used to amplify the product.

Step 2: Digestion of Vector

Gibson assembly reactions require approximately a 1:1 molar ratio of each fragment in the mix. I usually perform 10 µl total volume reactions using 5 µl of 2X HiFi assembly mix, and 5 µl of DNA. I usually use 50-75 ng of vector per reaction. Because the total volume of the reaction is small and multiple DNAs are often in the reaction it is important to have the vector relatively concentrated (at least 100 ng/µl final concentration). I perform digests as described in digestion of DNA products, but using 2 µg of vector in a 100 µl reaction volume and 2-3 µl of each restriction enzyme. I incubate the reaction for two hours to drive the reaction to completion. If a unique restriction site is present in the vector between the two sites being used for the assembly reaction, I will often add that enzyme to the mix to reduce the possibility of having significant amounts of single cut vector (which religates and increases background). After column purification (step 4) this will yield 10-20 reactions worth of cut vector which can be stored for later use.

PCR can be used as an alternative to create a vector. In that case, use a very small amount of vector as template and perform a DpnI digestion to cleave the template before purification of vector.

Step 3: PCR amplification of inserts

Perform as described in PCR amplification of insert DNA in the restriction enzyme cloning section. A 25 µl reaction is usually sufficient, but if many inserts are being used, and the PCR is inefficient, a larger reaction may be needed to obtain product that is concentrated enough for the assembly reaction.

Step 4: PCR clean up and purification of vector and insert

Perform as described in Clean-up purification of PCR Products in restriction enzyme cloning section. Each insert and the vector is purified and eluted in 10 µl to maintain as high a concentration as possible. It is important that primer dimers not be present as these will serve as homology templates and greatly reduce the efficiency of the reaction. Gel purification can be used to purify a fragment away from primer dimers. If one is adding many inserts, then the DNAs need to be relatively concentrated to permit the mixing of all of the DNAs in a small volume.

Step 5: Hi-Fi reaction

Mix DNAs for the assembly reaction

50-100 ng of vector (~0.025- 0.05 pMoles vector = 100 ng of 3 kb plasmid =0.05 pMoles)

20 to 100 ng of each insert (aim for ~0.05 pMoles of each insert[30]).

Total volume of all DNA should be <= 5 µl.

Add equal volume of 2 X HiFi mix.

Incubate at 50°C for 15 min to 1 hr.

Step 6: Transformation in DH5α

Perform as described in Transformation of *E. coli* with 1 µl of the reaction volume.

Typical reactions will yield a few hundred colonies.

Step 7: Analysis of clones

Pick 4-6 colonies to grow up and isolate DNA using a mini-prep protocol and analyze with restriction digestion. For clones created from small numbers of inserts (<5), the vast majority of colonies are usually correct if the vector was well digested. For larger assemblies the frequency of correct clones will sometimes be lower.

Troubleshooting

No colonies

The most likely possibilities are:

- Poor efficiency competent cells.

Clones are partial assemblies missing a specific fragment

The most likely possibilities are:

- Insert is contaminated with a primer dimer assembly that is used in place of the insert in the reaction

Clones resemble input vector

The most likely possibilities are:

- Vector was not completely digested.
- Sample was not well mixed and some vector did not “see” enzyme.

Clones resemble other plasmid

The most likely possibilities are:

- that an insert was prepared from a plasmid template with the same drug selection marker as the final product, and it was not cleaved or removed during insert purification.
- A *ccdB* negative selection vector was used, and spontaneous loss of the *ccdB* occurred during preparation of the vector.

[30] Very small fragments (<200 bp) need to be added in higher quantity.

Site directed Mutagenesis

Overlap PCR method

This method is basically the combination of amplification of a product by overlap PCR followed by standard restriction enzyme cloning. This protocol has fallen out of favor as higher fidelity polymerases have become available, and as the cost and ease of sequencing has dramatically reduced. The advantage of this protocol is that only the PCR product is actually amplified greatly lowering the chance that the remainder of the plasmid has nucleotide changes.

Step 1: Design of oligonucleotide

Identify unique cloning compatible restriction sites closest to the lesion site that span the site.

Design oligonucleotides that will amplify this fragment, called outside primers L and R (Figure 8).

Design a pair of primers with the targeted mutation be included into both primers, called inside L & R.

The mutation should be in the middle of the insert L an R primers, if possible.

The mutation should be at least 12-15 bases from the 3-terminus.

Design your primers (including the mutations) to have a T_m >=78°C.

Step 2: PCR amplification of left and right inserts

Amplify the left and right products using inside L & outside L, and inside R & outside R, as described in PCR amplification of insert DNA in the restriction enzyme cloning section.

Step 3: Clean up purification of each PCR product

Clean up the left and right PCR products as described in Clean-up purification of PCR Products in the restriction enzyme cloning section.

Step 4: PCR overlap amplification of lesion

Add in order on ice

17- x - y µl H20
5 µl 5X Q5 buffer
2 µl 2.5 mM dNTPs
x µl PCR left product (50 fmol)
y µl PCR right product (50 fmol)
0.5 µl outside primer A (25 µM)
0.5 µl outside primer B (25 µM)
0.3 µl Q5 polymerase (usually overkill)

Start PCR machine, and move tube to machine after it has reached 72°C.

Amplify using appropriate conditions for the length and annealing temperature of the oligonucleotides.

Step 5: Subclone fragment into the parental vector

Subclone the PCR fragment into the parental vector as described in restriction enzyme cloning.

Two primer DpnI mediated mutagenesis using Phusion® polymerase[31]

Step 1: Design of mutagenesis primer design

The targeted mutation should be included into both primers.

The mutation should be in the middle of the primer, if possible.

The mutation should be at least 12-15 bases from the 3-terminus.

Design your primers (including the mutations) to have a T_m >=78°C[32].

If possible design the lesion such that a restriction site is altered to simply identification of mutant.

The extent of overlap among the two oligonucleotides will effect which polymerases can perform this reaction (see Xia et al. 2015). The protocol assumes Phusion is being used.

Step 2: PCR amplification

mix in order

17 µl	water
5 µl	5X Phusion Buffer[33]
2 µl	dNTPs (2.5 mM each)
1/2 µl	Primer F (25 µm)
1/2 µl	Primer R (25 µm)
0.5 µl	Template DNA (10-20 ng)
0.5 µl	Phusion Polymerase

Amplify using following protocol

98°C 0:30 (20 X 98°C 0:10, 60°C 0:30, 72°C 1:00/per kb of plasmid) 72°C 5:00

Add 1/2 µl DpnI to PCR and incubate 1 hr at 37°C

(optional) run 2-5 µl of PCR on a gel. If a PCR product is detect at the expect size of the plasmid, the mutagenesis is highly likely to work.

Step 3: Transformation of *E. coli*

Perform as described in Transformation of *E. coli* with 1 µl of the reaction volume.

You should get hundreds of colonies if the plasmid is <10 Kb.

Step 4: Analysis of clones

We usually grow up 4 colonies and prepare DNA using our standard mini-prep protocol. Screen the transformants for the desired mutation using restriction digest or sequencing depending on the lesion being created.

Notes

Efficiency

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[31] Phusion® polymerase is a commercial version of Pfu-S (Wang et al. 2004) created by fusing Sso7D DNA binding protein from Sulfolobus solfataricus to Pfu Polymerase. Phusion® can be purchased from NEB. It can also be purified easily.

[32] This recommendation is complicated since Tm is calculated differently by different programs.

[33] We used to use Pfu polymerase, then it started working much less well. We suspected a change in the formulation of the enzyme. We switched to Ex-Taq, which also works. Currently, Phusion® is the generally recommended polymerase.

This protocol is usually highly efficient. Greater than 90% of colonies should be correct.

Plasmid size

We have used the protocol for plasmids over 18 kb in length. Plasmids below 10 kb seem to work routinely. In most cases, a vast majority 80-100% of colonies are correct. For longer plasmids it may be necessary to work with PCR to optimize synthesis.

This protocol fails completely using Q5 polymerase if the oligonucleotides overlap extensively. However, a recent publication demonstrated that it works robustly if one designs oligos that only partially overlap. The efficiency was highest when the oligonucleotides that overlapped approximately 20 bp, and had 10 base extensions (Xia et al 2015).

Megaprimer multi-lesion method

Step 1: Design mutagenesis primer(s).

Only a single primer is ordered for each lesion.

The primers are designed to be in opposite orientations, and create a PCR product that contains both lesions which is then purified and used as the primer for the “mutagenesis”.

The targeted mutation should be in the middle of the primer.

Design your primers (including the mutations) to have a T_m >=78°C.

If possible design the lesion such that a restriction site is altered to simply identification of mutant.

Step 2: PCR amplification

mix in order

- 17 µl water
- 5 µl 5X Phusion Buffer[34]
- 2 µl dNTPs (2.5 mM each)
- 1/2 µl Primer F (25 µm)
- 1/2 µl Primer R (25 µm)
- 0.5 µl Template DNA (10-20 ng)
- 0.5 µl Phusion Polymerase

Amplify using following protocol

98°C 0:30 (20 X 98°C 0:10, 60°C 0:30, 72°C 1:00/per kb fragment size) 72°C 5:00

No DpnI digestion is need if the template is the same as the template being used for the final mutagenesis.

Step 3: Clean-up purification of PCR Product

Add 150 µl 1:9 PBI to PCR reaction (5-6 volumes of PBI).

Load onto Qiagen mini elution column.

Spin 30 seconds to bind to column. Remove binding liquid.

Add 300-500 µl PE buffer, spin 30 sec. Remove PE.

Add 300 µl PE buffer, spin 30 sec. Rotate tube 180 degree, Spin 1 minute.

Transfer column to new 1.5 ml Eppendorf tube.

Add 10 µl of TE. Spin at 0.1 RCF, 1 minute to drive TE into column.

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[34] We used to use Pfu polymerase, then it started working much less well. We suspected a change in the formulation of the enzyme. We switched to Ex-Taq, which also works. Currently, Phusion is the generally recommend polymerase.

Spin at full speed 1 minute.

Yield is usually about 1-1.5 µg of DNA in 10 µl.

Step 4: PCR of final product

mix in order

17 µl water
5 µl 5X Phusion Buffer[35]
2 µl dNTPs (2.5 mM each)
2 µl PCR product (100-200ng)
0.5 µl Template DNA (10-20 ng)
0.5 µl Phusion Polymerase

Amplify using following protocol

98°C 0:30 (20 X 98°C for 0:10; 60°C for 0:30; 72°C for 1:00/per kb of plasmid) 72°C for 5:00

Add 1/2 µL DpnI to PCR and incubate 1 hr at 37°C

(optional) run 2-5 µl of PCR on a gel. If a PCR product is detected at the expect size of the plasmid, the mutagenesis is highly likely to work.

Step 5: Transformation of E. coli

Perform as described in Transformation of *E. coli* with 1 µl of the reaction volume.

You should get hundreds of colonies if the plasmid is <10 Kb.

Step 6: Analysis of clones

We usually grow up 4 colonies and prepare DNA using our standard mini-prep protocol. Screen the transformants for the desired mutation using restriction digest or sequencing depending on the lesion being created.

Single primer multi-lesion method

This approach is based on building a full copy of the plasmid by ligation of partial primer product using Taq ligase, a high temperature ligase. The protocol included here comes from Tom Richard's lab at Penn State https://openwetware.org/wiki/Richard_Lab:Site_Directed_Mutagenesis and is based off of the Quickchange multi-site directed mutagenesis kit (Stratagene). It requires a polymerase that does not strand displace and is less reliable than dual primer mutagenesis.

Step 1: Design mutagenesis primer(s).

Only a single primer is ordered for each lesion.

The protocols all call for all primers to be in the same orientation.

The targeted mutation should be in the middle of the primer.

Design your primers (including the mutations) to have a T_m >=78°C.

If possible design the lesion such that a restriction site is altered to simply identification of mutant.

---

[35] We used to use Pfu polymerase, then it started working much less well. We suspected a change in the formulation of the enzyme. We switched to Ex-Taq, which also works. Currently, Phusion is the generally recommend polymerase.

Step 2: Mutagenesis PCR mix:

set ip in oder on ice:

16 µL Water
1.5 µL DMSO(100%)
0.5 µL MgSO4(50 μM)
10 µL Phusion HF Buffer
5 µL T4 Ligase Buffer
5 µL 10x Taq Ligase Buffer
0.5 µL Each Mutagenesis Primer (at 40 μM)
2 µL dNTPs
2 µL Template
2 µL PNK
2 µL Taq Ligase
1 µL Phusion Polymerase
1 µL reverse orientation primer anywhere on plasmid (optional)

PCR conditions

37°C for 30 minutes (to kinase oligos) followed by

95°C 3:00, 20 x (95 °C 1:00, 55°C 1:00, 65 0:30 sec/kb of kb length

Add 1µL DpnI restriction enzyme and digest 1 hr.

Step 3: Transformation of E. coli

Perform as described in Transformation of *E. coli* with 1 µl of the reaction volume.

You should get hundreds of colonies if the plasmid is <10 Kb.

Step 4: Analysis of clones

We usually grow up 4 colonies and prepare DNA using our standard mini-prep protocol. Screen the transformants for the desired mutation using restriction digestion or sequencing depending on the lesion being created.

Notes

Some protocols for this multi-primer approach use a reverse orientation primer in the backbone of the vector. This is presumably to create a double stranded final template. Given that this is the case, it is unclear if ordering all mutagenic primers in the same orientation is logical or not. We have only tried this protocol to incorporate two lesions using primers in the same orientation without a reverse orientation primer. The idea here is that single stranded circles are the functional DNA being transformed into E. coli.

This protocol is less efficient than the single site mutagenesis protocols. Only ~ 25-50% of clones incorporate all the lesions.

Isolation of Plasmid DNA

We use silica column mini-preps to isolate virtually all of the DNA we use in the lab. They work reliably for PCR, restriction digestion, ligation, RNA synthesis, injection into zebrafish and injection in to *C. elegans*. Note that we do not work with mammalian tissue culture, and I cannot comment of the efficacy of these DNA preps for work in that system. The preps are quick, reliable and very inexpensive (in part because we reuse the columns and make our reagents for the preps). Under very rare circumstance we will prepare DNA using a midi-prep, but I do not detail this procedure.

Growing the cultures

Inoculate a 1.5 to 5 ml culture with a colony[36]. Grow overnight at 37°C. We find it unnecessary to add antibiotics to these cultures. Adding antibiotics increases yields only modestly under most conditions (especially for Amp^R plasmids).

Mini-preps

Collect 1.5 ml of cells[37] in an Eppendorf tube. (Save the rest to freeze down the clone).

Spin 30 seconds in microfuge at full speed and remove media.

Add 250 µl of Buffer P1[38]. Resuspend cells.[39]

Add 250 µl of P2 buffer. This lyses the cells.

Mix by closing tubes and rocking upside down two times.

Add 350 µl of N3 buffer to precipitate much of the cellular material.

Mix by closing tube and rocking 3 to 4 times.

Spin 10 minutes in microfuge at full speed in the cold room[40].

Transfer 750 µl of supernatant to a clean blue Qiagen column[41]. Spin 5 seconds at full speed.

Remove the supernatant from the collection tube.

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[36] We often grow ~ 3 ml. 1 ml to freeze the stock at -80 by addition of DMSO to 7% (78 μl DMSO for 1.0 ml of culture).

[37] The newest Qiagen mini-prep columns saturate at about ~20 -25 µg DNA, and for many 4-10 kb plasmids, DNA isolated from 1.5 ml fresh culture will saturate the column. Using large volumes of culture in these cases is counter productive. For lower copy number plasmids, using up to 5 ml of culture can increase yields. For lower copy number plasmids, perform parallel lyses of 3-5 ml aliquots of a culture and load multiple reactions on a single column to further increase yield. The P2 lysis solution just cannot handle more that 3-5 ml of culture. The highest yields come from inoculation of large fresh colonies that are grown ~ 12 hr. Usually, even 20 hr incubations at 37°C still yield reasonable DNA. Very long culture growth will sometimes lead to lysis of the culture especially if the incubator temperature is high (>=38, or the plasmids are very large. If lysis occurs, the culture often regrows. In such cases the culture will be less dense than expected and flocculant material will likely be visible in the culture. Such cultures will yield exceedingly low levels of plasmid.

[38] Use a pipetter, or if doing many preps, one can use an Eppendorf multi-dispenser.

[39] Options are pipetting up and down (when doing a few preps), vortexing (good for medium numbers), or placing tubes on an Eppendorf Thermomixer R shaker for 5 minutes (good when doing many).

[40] RT works also. We do this spin in the cold room to reduce the noise in the lab.

[41] One wants to avoid the white material that sometimes comes off the wall of the tube. Make sure the tube is oriented with that side down, and remove slowly. If large amounts of white precipitate come up, one can place the liquid in a clean Eppendorf and re-spin. Waiting more than a few minutes after the 10 minute spin before removing exacerbates this problem.

Add 750 µl of PE buffer to the column. Spin 5 seconds at full speed.

Remove the PE buffer from the collection tube. Spin 30 seconds at full speed.

Rotate tubes 180 degrees in the centrifuge (this reduces dead volume in the column).

Spin 1 minute.

Transfer the column to a 1.5 ml Eppendorf tube.

Add 65 µl of TE to column[42].

Wait 1 minute, then spin 1 minute. Transfer column back to the collection tube.

Store DNA at 4°C . These preps are stable for years as long as EDTA is in the elution buffer.

Use a NanoDrop spectrophotometer (or equivalent) to get an accurate concentration.

Typical concentration will be 100-300 ng/µl depending on the vector backbone, size, culture volume, etc.

Very small and very large plasmids usually have lower yields.

Recycling columns

Many people are shocked that we would reuse Qiagen columns. I have reused the same columns > 20 times. Yields do not decrease appreciably in this period of reuse. Reusing columns does require some caution. A very very small amount of contaminating DNA can remain from the prior use even after our column clean-up. However, for virtually all applications this is not relevant. But in a few cases it is. I use new columns when I think a very small amount of contamination might cause problems in my experiments. A cautious way to re-use columns is to use them for diagnostic, but not preparative work. When you are identifying a clone of interest, you use recycled columns to identify the clone. But, you re-prep the same stock using a fresh column to create a working stock. This approach works great if your efficiency of identifying correct clones is low. Once > 50% of the colonies you prep in cloning experiments are correct, then this starts to become disruptive to the flow of experiments. I balance efficiency with purity when deciding whether to use new or recycled columns. I rarely use new columns. I have yet to have an experiment knowingly harmed from using recycled columns. But, I readily admit that it formally poses significant risk for disruption of both cloning experiments and downstream *in vivo* experiments that use the prepared plasmid reagents. My perspective is that this risk is negligible in the hands of a thoughtful experienced researcher.

There are three ways we recycle columns. If one recycles them using a “safe” protocol, then one can treat the column as new. The “safe” method uses 1 M HCl to the column to break DNA remaining on the column into small fragments. However, for most applications, a simple long incubation with a 400 µl of TE will clear the column of >99.9% of the prior plasmid purified in the column. Another approach that works is autoclaving the columns.

Standard Column Cleaning:

Add 400 µl of TE to column. Increasing this to 750 µl will reduce the chance of slight contamination due to reagents remaining on the column sidewall. Incubate 5 min to overnight (if you forget about them) at RT.

Spin column 10 sec in a microfuge at full speed to clear column.

Remove liquid from collection cup. Store columns at RT for next use.

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[42] We use TE (10 mM Tris pH 8.0 , 0.1 mM EDTA instead of with EB (elution buffer) that comes with the Qiagen Kit. EB does not have EDTA in it, and if you use this your DNA will degrade much much faster. The EDTA chelates any residual Mg2+ which an essential co-factor for DNAses. The amount of EDTA is so small it will not interfere with DNA restriction digestion and PCR reactions as it is swamped out by the Mg in the reaction buffers.

Safe Column cleaning:

Add 150 µl of 1M HCl to the column. Incubate 1hr at RT.

Spin in the microfuge for 10 seconds to clear column.

Add 150 µl of 1M NaOH. This neutralizes the HCl.

Spin to clear column.

Add 200 µl of TE to column.

Spin 10 seconds at full speed to clear column.

Remove all liquid from the collection cup and store column at RT for next use.

The above protocol ignores possible DNA contamination on the upper sidewalls of the column. This protocol will destroy all contaminating DNA in the silica bead, but not DNA on the sidewalls of the column.

Super Safe Column cleaning:

This protocol will destroy all DNA in the column, but requires removing 1M HCl from the collection tube.

Add 750 µl of 1M HCl to the column. Incubate 1 hr at RT.

Spin in microfuge for 10 seconds at full speed to clear column. Remove HCl from collection tube.

Add 750 µl of TE to the column. Spin 10 sec to clear column.

Add 750 µl of TE to column. Spin 10 seconds at full speed to clear column.

The two TE spins neutralize any remaining HCl.

Remove the TE from the collection tube and store column at RT for next use.

Autoclaving Column cleaning:

Rinse column using the standard protocol, then autoclave for 15 minutes using a dry cycle. We have demonstrated that even if one autoclaves a column that is saturated with plasmid DNA, that after autoclaving no colonies can be recovered by DNA transformation into *E. coli*. However, it may still be the case that sufficient small fragments of DNA are present such that one could PCR amplify fragments from solutions eluted from an autoclaved column. Also note that a little ring that hold down the column material in the bottom of the column sometimes becomes dislodged by autoclaving. It can easily be push back down using a pipette tip. We have not noticed that this effects the functionality of these columns.

These protocols can be also be used to recycle PCR purification columns.

Long Term Storage of Plasmid clones

Small mini-preps of plasmid DNA are stable for several years at 4°C in TE. That is the approach we take to short and medium-term storage of plasmid DNA. Note that Qiagen and other companies do not put EDTA in their elution buffer, and this will greatly reduce the stability of this DNA (just add EDTA to 0.1 mM to their elution buffer). The evaporation condensation cycle in the frost free 4°C refrigerators will take their toll on small volume samples. Storage at -20°C, especially if the freezer is NOT frost-free will last longer (Frost-free freezers cycle several times a day to above freezing to remain frost-free). Freeze-thaw cycles damage DNA, but I am not sure whether the evaporation condensation cycle at 4°C or freeze-thaw cycles cause more damage. We haven't stored our DNAs at -20°C simply because we do not have extensive -20°C storage space.

To ensure that we do not lose our plasmids, we store a bacterial stock of the plasmid in the *E. coli* host at -80°C. We typical take 1 ml of culture and add DMSO to 7%. We typically do this at the time we make the

plasmid. These stocks stay viable for more than 20 years. One advantage of this, is that it is easy to re-streak the stock on a plate, and then grow up the bacterial strain to prep a new stock of DNA. Furthermore, even if the stock is no longer viable, a mini-prep of the content of the vial will yield quality DNA that can be re-transformed into *E. coli.* Thus, even after a -80°C freezer crash, if the stocks are refrozen, plasmid DNA can still be re-isolated from the inviable stocks.

Restriction Digestion Analysis of DNA

The most common way to analyze plasmid DNA is performing restriction digestion followed by gel electrophoresis of DNA. The method is quick and extremely reliable if performed correctly.

Restriction Enzymes

Restriction enzymes are proteins that cleave DNA usually at a specific sequence. Most restriction enzymes cleave at palindromic sites. For example, EcoRI cleaves DNA at the sequence G/AATTC, the slash being the position where the DNA is cleaves.

```
5′ NNNNNNNGAATTCNNNNNNN  3′      5′ NNNNNNG 3′        5′ AATTCNNNNNN 3′
3′ NNNNNNNCTTAAGNNNNNNN  5′      3′ NNNNNNCTTAA 5′        3′ GNNNNNN 5′
```

This results in two fragments with complementary single stranded ends. Different enzymes leave distinct overhangs (either with the 3' or 5' end extending), and yet other leave blunt double stranded ends. Restriction enzymes require Mg^{2+} to cleave, and a few also require S-adenosyl-methionine to cleave.

Unit definition of enzymes

Restriction enzymes are sold with specified activity. NEB usually defines 1 unit of enzyme as the amount of enzyme that will cut 1 µg of a defined template (often λ DNA) in 1 hr. One can calculate how much enzyme is needed to cleave the DNA of interest if one knows the number of sites in the target plasmid. For example, BglII has 6 sites in the λ DNA genome (50 Kb) . If one assumes BglII cleaves λ DNA and plasmid DNA with the same efficiency[43], and our plasmid is 8 kb and has two BglII site, there is a cleavage site in λ DNA 1 per 8kb, and 1 per 4kb in our plasmid, so it will take 2 units of enzyme to cut 1 µg of our plasmid. Rather than actually make this calculation, we usually simply add 1/2 µl of enzyme to our typical 10 µl 200 ng DNA digests. 1/2 µl is roughly the minimum one can reliably pipette with a P20 pipetter. This is usually overkill, but simple.

Digest setup

Add in order

- 8 µl H20
- 1 µl 10X Restriction Buffer
- 1 µl DNA (200 ng)
- 1/2 to 1 µl of Restriction Enzyme

Incubate at the suggested temperature for 1hr.

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[43] This assumption is not always valid. NEB lists enzymes that favor certain templates over other (plasmids are supercoiled and this can effect DNA binding and cleavage).

Master mix reactions

When multiple samples are being digested with the same enzyme, we mix water, 10X buffer and enzyme without template, and aliquot the reaction mixture to tubes containing only template DNAs.

General guidelines

Restriction digestions are generally simple to perform. In general the following will insure that digests work well.

1) Try to digest the minimum amount of DNA needed for an experiment.

2) keep DNA concentrations below 50 ng/µl final concentration.

3) Limit enzyme addition to than 10% of the total reaction volume.

4) Use the appropriate buffer[44] and temperature for the enzymes[45].

5) Mix well, and spin tube before incubation to make sure that all DNA contacts enzyme.

6) Small volumes reactions performed for long incubation times do not work well unless performed in a PCR machine with a heated lid (or under oil). Otherwise evaporation will cause problems.

Troubleshooting Restriction Digests

Some of the most common problems with restriction digests are:

1) old enzymes that are no longer active

Some enzymes will stay active 20 years- our RsaI restriction enzyme from NEB officially expired in 5/95, but still works well in 2017. Others lose activity quickly (even a few months if treated poorly). Test whether the enzyme remains functional by digesting a small amount of a high quality DNA. If the enzyme remains active, increase the reaction time if the enzyme is active for long periods of time (NEB has tables that list length of time different enzymes maintain activity and this varies from under and hour to greater than 16 hrs).

2) inappropriate reaction conditions

Of course, buffer and temperature are critical for high activity. But, some enzymes have other specialized conditions for high activity. Some are greatly aided by BSA, some require two sites in the plasmid to cut effectively, a few require SAM. Check details for the specific enzyme.

3) using too much DNA, or poor quality of template DNA.

Mini-prep purified DNA can be contaminated with ethanol (poor spinning after washes), RNA (old RNAse) or cell debris (cleared lysate wasn't clear). Try digesting less DNA, or re-prep the DNA.

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[44]NEB Buffer formulation (1X)

Buffer 1.1	no salt,	10 mM Tris-propane HCl pH 7.0,	10 mM $MglCl_2$, 100 µg/ml BSA
Buffer 2.1	50 mM NaCl,	10 mM Tris-HCl pH 7.9,	10 mM $MglCl_2$, 100 µg/ml BSA
Buffer 3.1	100 mM NaCl,	50 mM Tris-HCl, pH 7.9	10 mM $MgCl_2$, 100 µg/ml BSA
Smartbuffer	50 mM KAc ,	50 mM Tris-Ac pH 7.9,	10 mM $MgAc_2$, 100 µg/ml BSA

[45] If two enzymes are not compatible in the same buffer they can be added sequentially. In these cases, start with the lower salt concentration enzyme after performing the first digest, add salt to increase concentration to the higher buffer.

PCR

The Polymerase Chain Reaction (PCR) is one of the most widely utilized methods in modern molecular biology. It is a rapid, robust, and reliable method for selectively amplifying and synthesizing microgram quantities of specific DNAs from a complex mixture.

How PCR works

PCR synthesizes DNA by repeated steps of denaturing DNA, hybridizing short oligonucleotide primers to the denatured DNA, then synthesizing a new DNA stand using the 3' end of the oligonucleotide to extend the new strand of DNA. 30 to 35 cycles of DNA denaturation (at ~95°C), oligonucleotide annealing (at ~ 55°C), and DNA synthesis (at ~ 72°C) yield microgram quantities of DNA from even a single molecule as the template. The reactions are typically performed in a 200 µl PCR tube in a cycling machine with a heated lid to prevent problems of evaporation and condensation during the cycling [46].

Technical aspects of PCR polymerases

There are many different high temperature polymerases available for purchase for distinct uses. For cloning, where one wants at all cost to avoid errors, we usually use Q5 polymerase from NEB because it is very robust and has extremely high fidelity. But it is expensive. If Q5 fails to work, PrimeStar from TaKaRa is another robust, high fidelity enzyme. For the analysis of large DNA insertion in the genome, Long Amp from NEB is what we have found to be most effective, often being able to robustly amplify 10-15 kb fragments from high quality genomic DNA. For diagnostic PCR (scoring alleles in worms, etc), use the cheapest polymerase that will work reliable for the assay. Often this is our own homemade Pfu-ssoD7[47] polymerase (Wang et al. 2004) and other times we use Ex-Taq (Takara Bio). If the only goal is to use PCR as an assay (and one is not creating a DNA clone for further use), then the choice of polymerase boils down to what amplifies products most robustly at the cheapest cost.

Different commercial polymerases recommend different cycling conditions. These can have a significant impact of the effectiveness of the enzyme. For example, Q5 polymerase recommends a very high temperature denaturation (98°C), and a very high annealing temperatures (3°C > T_m of the oligonucleotides). By contrast, PrimeStar recommends lower denaturation temperature (95°C) and short annealing times at lower temperatures. These recommendations are based on the thermostability of the polymerase, and the activity of the various polymerase and exonuclease activities of the polymerases. Changing parameters can have dramatic effects on the outcome!

Designing oligonucleotide primers for PCR

General guidelines

In general, oligonucleotides will be most effective if they have a T_m of above 55°C. I usually aim for a T_m of 58°-60°C [48]. First, identify a stretch of DNA where you would like the primer to anneal. If possible, avoid highly GC rich, or highly AT rich stretches, and long repeats of the same nucleotide (>5). However, if one is targeting a specific site in the genome you may have little choice in designing the sequence except for how long to make the primer. I try to clamp the 3' end of my oligonucleotides with a G or C, if possible. I try to avoid palindromes

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46 In absence of a heated lid, the reactions are done under mineral oil.

47 Phusion® polymerase

48 The true Tm of a primer is based on salt concentration, Mg^{2+} concentration and primer concentration (and other assumptions like pH). There are several different methods for calculating a Tm, some which take these into consideration, and others that don't. Multiple web sites have calculators for determining Tm. I usually use the ApE program Tm. One can set the primer concentration and salt concentration for calculation of Tm in the Primer selection window in ApE. The ApE formula is not accurate for very long primers. Other options include: http://www6.appliedbiosystems.com/support/techtools/calc/, http://biotools.nubic.northwestern.edu/OligoCalc.html, http://www.biophp.org/minitools/melting_temperature/demo.php or the NEB Tm calculator https://tmcalculator.neb.com/#!/main.

at the 3' end of the primer, as these will form primer dimers more readily. After having identified a good position for the 3' end, extend or shorten the 5' to to get the Tm you desire (we use ApE which displays the Tm of a selected region of DNA to perform this analysis).

Restriction sites near ends of primer

I then add the restriction site(s) on the 5' end, making sure I keep frame if relevant. Then I add 3 or 4 base to the 5' of the restriction site being introduced. Many enzymes do not cut well when binding to the exact end of DNA. NEB has a table that lists the behavior of many enzymes when cutting close to an end.

Other sequence features

Also consider whether adding a second restriction site internal to the restriction site being used for this cloning step if it might help for later cloning steps (for example to add a tag to the gene later). Consider other modifications you might introduce (e.g. modifying the Kozak/Shine-Delgarno ribosome binding site, or adding a second stop codon).

Length and purification of primers

We try and order primers at the 25 µmole scale <= 60 bases from IDT. They are cheap and very reliable. Most primers that we we order we use only once to create a specific modification. If needed, we will order primers between 61-90 bp from IDT at the 100 µmole scale. These cost 50% more than the smaller primers, but are still usually very reliable. Under no circumstances do we ever have these primers purified for the general purpose of cloning. The cost is prohibitive and the benefit is minimal.

Primer Design programs

Many programs are available to select appropriate high quality PCR primers[49]. These definitely can improve oligonucleotide performance, but I rarely find they provide a measurable benefit when the time required to use them is incorporated into the equation. There are three reasons for this. First, 1) my experience is that >95% of primers designed purely by eye work well. This makes spending the effort to use primer design programs non-beneficial in the vast majority of the cases. Second, in many cases, the design of the primer is very much defined by the position at which one wants to introduce a lesion, or add a restriction site. In all of these cases, it is not possible to move the position of the oligonucleotide to make it "better". In these cases, there is little utility to those programs. Third, when oligos don't work, it is often not the oligonucleotide per se causing the problems, but rather often it is the structure of the DNA being amplified. Often the cause is highly repetitive, or very GC rich DNA.

Storage of oligonucleotide primers

We store our oligonucleotide stocks at -20°C as 100 µM master stocks, and as 25 µM working stocks. We resuspend our oligonucleotides in water. We have 20 year old stocks that have been stored this way that still work fine for PCR. We also have a database that lists all of the oligonucleotides that have been ordered in the lab. The file is used to search plasmid sequences using the ApE DNA analysis program when we are searching for a primer (for diagnostic PCR or sequencing).

Our lab used to store oligonucleotides that were used together in reactions premixed as 25 µm stocks. Both the plus and minus strand oligo for a common reaction were kept as a 25 µm stock. We have found that unless these are preheated to > 72°C before aliquoting to a PCR reaction, they can often greatly increase primer dimer formation. What is likely happening is that the two primers are forming bimolecular annealed complexes while stored together at low temperature. We now usually keep our forward and reverse primers working stock separate even for assays we are performing regularly in high volume.

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[49] Primer-BLAST at NCBI will suggest primers and test if they are specific by blasting against an organism sequence of choice. https://www.ncbi.nlm.nih.gov/tools/primer-blast/ . ApE will also select primers under defined set of conditions (length, annealing temperature, % GC, GC clamp, consecutive bases, self complementarity and heterodimer formation)

PCR reaction hot and cold starts

One major problem with PCR is non-specific annealing of primers to template DNA, or to primers during the reaction set up. The can lead to large quantities of primer dimers and spurious products in a reaction. Two approaches can be used to minimize these. The most effective is a “hot start”. In this case, the polymerase is added to the reaction after the reaction has been heated to denaturation temperatures, to prevent the low temperature annealing complexes from forming and yielding products. One simply adds the enzyme to the reactions after a first heat cycle. However, this take significant extra effort and can be complicated when performing many reactions.

Some commercial enzymes sell special formulations of their products that include an inhibitor that prevents polymerase activity at low temperature (usually an aptamer that inhibits the polymerase that is temperature reversible). These work well, but are expensive.

A third approach, which is less reliable, but one we find to be a reasonable compromise, it to set up the reactions on ice, then preheat the PCR machine and transfer the reactions directly from ice to denaturation temperatures. We call this a “cold start”.

PCR reaction set up

We typically find 25 µl reactions are sufficient to amply sufficient product for most cloning reactions.

Individual reaction using Q5 polymerase

Add in order on ice

17 µl H20

5 µl 5X Q5 buffer

2 µl 2.5 mM dNTPs

1 µl template

0.5 µl primer A (25 µM) Tm 59°C

0.5 µl primer B (25 µM) Tm 59°C

0.3 µl Q5 polymerase (usually overkill)

Start PCR machine, and move tube to machine after it has reached 72°C.

Amplify using appropriate conditions for the length and annealing temperature of the oligonucleotides

For Q5 polymerase this would be 98°C for 30 second followed by 30 cycles of 98°C for 10 seconds, 62°C for 30 seconds , and 72°C for 0.5-1.0 min / kb of final product. If the oligonucleotides have a different Tm, use 3°C above the lowest Tm for Q5 polymerase.

Multiple reactions

When performing multiple reactions for diagnostics often 12.5 or 15 µl reactions are sufficient. Our standard reaction set up conditions involve making a master mix (on ice and in order) of water, buffer, dNTPs, and primers. We then place templates of interest in individual tubes in PCR strips and place these on ice. We then add polymerase to the PCR reaction, mix, and aliquot the mixture to the template containing PCR strip. The strips are capped, spun quickly in a table top micro-microfuge, and placed back on ice. We start our PCR machine and add the strips to the machine after the first step has reached above 72°C.

Reaction volume effects

We have noticed that PCR reactions often do not scale. Conditions that work for 14 -25 ul often work less well for 50 ul reaction and fail for higher volumes. This likely is due to the time required to heat and cool larger volumes of liquid. If you need to scale up scale up by running more reactions of the same volume (e.g. 8 x 25ul), rather than increase the volume of individual reactions (2 X 100 ul).

Troubleshooting PCR

There are generally two types of problems with PCR. 1) Too many products, or unexpected products. 2) No product or little product.

Contamination

Contamination is a significant problem with PCR. Even a tiny amount of contaminating template can cause havoc to PCR. A no template control is a simple method for identifying contamination in any component of the reaction except the template itself - if that is contaminated with another template. Defining the source of contamination can be difficult, as it can not only be the reagents, but also pipette men due to poor technique of pipetting. I recommend just replacing the reagents. If the event occurs repeatedly, then start testing individual components to determine the source of contamination, and try to deduce the mechanism by which it is become contaminated.

Too many bands including predicted correct product

Contamination is a potential source for unexpected products. But, if not due to that, this problem is usually because either the oligonucleotide(s) are annealing at another location in the template, or that some repeated element is creating alternatively annealed dsDNA templates or a GC rich region is causing some template skipping.

1) Reduce the amount of template 2-3 fold and raise annealing temperature 4°C and repeat.
2) If still problematic, perform a gradient PCR.
3) Try a PCR enhancer like the GC enhancer (provided with Q5 polymerase) or betaine to a final concentration of 0.8 to 1.6 M in the PCR reaction.
4) Try another polymerase.
5) Order longer oligonucleotides (and raise the T_m for new reactions).

Products of unexpected size, but no proper product

Contamination is often the cause here. If it is not contamination, the difference between seeing the expected product (see above) and not seeing strongly suggests the possibility that either the template is not as expected or oligos are not properly designed.

1) Double check the oligonucleotides.
2) Make sure that the template is correct especially if it is a clone and not genomic DNA.
3) Make sure oligonucleotides don't have alternate binding sites in the template nearby that could yield alternate products. A search using the template in ApE allowing 3 mismatches is a good start. if genomic DNA is being used, blasting the oligonculeotide against the whole genome can identify primers that bind to repetitive sequences in the genome.
4) If amplifying a genomic fragment, look for repeats in the genomic region between the oligos. These can cause the denature product to anneal between different copies of the repeat and either enlarge or shrink the size of the amplified product. Of course, the short products are favored in such a scenario.
5) troubleshoot as if too many bands.

High levels of primer dimers (bands below 100 bp)

Bands below 100 bp are usually the result of primer dimers resulting from primers annealing to each other and forming a very small PCR product. This can be the result of a single oligonucleotide hybridizing to itself, or to a second oligo.

1) Try a hot-start for the PCR.
2) Increase annealing temperature.
3) Check if oligonucleotides hybridizes at multiple sites in the template using BLAST or other programs.

4) Test if the product is independent of template. Formation of products is often independent of template if they results from primer-primer products.

No product at all

Three common sources at least:

1) oligonucleotides are not properly designed.

This is the most common problem. Check the oligonucleotides to make sure they actually anneal to the template you amplified. One might design a cloning strategy using a clone of a gene, and PCR from genomic DNA (and the clone might differ from the genomic version of the gene). Ordering the wrong strand for the reverse oligo is a common error.

2) Template is not correct.

Check the template using any means possible. Restriction digestion of a plasmid. Test the quality of genomic DNA by gel electrophoresis (is it intact or degraded?) or by PCR using oligonucleotides that are known to amplify products from the source of DNA.

3) A reaction component has gone bad, is contaminated, or was omitted from the reaction.

4) Less commonly, it is just a really difficult region to PCR.

To test this out, try ordering oligos that subdivide the product into two fragments. Test the left half and right half PCRs. If both of these work, it is likely just a very difficult region to amplify

a) increase extension time.

b) Add more polymerase.

c) Use GC enhancers.

d) Try a different polymerase.

5) An oligonucleotide is annealing poorly. In cases where one is using an oligonucleotide that has multiple mismatches with the template (e.g. lesioning an sgRNA site in a knock-in template), reducing the temperature of the first few cycles of the reaction may help significantly. For example, perform the first 5 cycles with an annealing temperature of 55°C, then then last 25 cycles with an annealing temperature of 65°C.

Specialized PCR protocols

Gradient PCR

Many PCR machines offer the option of having the PCR block form a gradient across one dimension of the block (usually the 12 tube direction). The most common use of this is to run a set of 8-12 reactions with a gradient of annealing temperatures from ~ 50°C to 70°C to identify the optimal annealing temperature for an oligonucleotide pair in a reaction.

Extending synthesis reaction time

Many PCR machines also offer the option of changing incubation time each cycle. The most common use of this is during PCR of long products. Since the polymerase is not completely stable during the denaturation, in later cycles the amount of polymerase becomes less capable of performing the extension. One way to counter this is to increase the extension time by 5-15 seconds each step.

Touchdown PCR

Although much less commonly used in the era of genomics, touch down PCR was a method devices to enrich for the most homologous annealing of oligos to a template when the sequence of the template is not known. For example to amplify a homolog from a non-sequenced genome using highly conserved regions to

design degenerate oligonculeotides[50] that could amplify the gene (or a chunk of the gene). Touch down PCR starts with a high annealing temperature and lowers it by 0.5 -1°C per cycle to favor more homologous products (the start amplifying in earlier cycles), while permitting the “search” for less homologous targets.

[50] Degenerate oligos have a mixture of multiple bases as some positions in the oligonucleotide. For example, if one expects a protein to encode Met-His-Gly-Glu-Glu-His, the oligonucleotide ATG CAY GGN AAR AAR CAY would anneal to the sequence at some frequency (Y=T,C; R= A,G; N=A,C,G,T).

Transformation of *E. coli*

There are many ways to make *E. coli* competent to take up DNA. The two general strategies used are electroporation and chemical competent cells. My preference is for the chemical competence method[51]. In addition to choosing the methodology, one must also choose the genotype of the *E. coli* to transform. There are many different strains available with different benefits and drawbacks. We use almost exclusively DH5α because it transforms well, grows reasonably fast, and we find most plasmids are relatively stable in the strain. Many other options are available for specialized purposes including working with very large plasmids, *ccdB* containing plasmids, protein expression, making ssDNA from plasmids with F1 origins, growing RK6 origins, and altering the copy number of fosmids[52].

Preparing Frozen Competent cells

This protocol is relatively simple and highly efficient. It is based on Hanahan (1983). The fact that the cells are grown at low temperature greatly increase time window and OD range during which the cells will prep at high efficiency (Inoue et al., 1990). Cells prepared this way standardly have a competency over 10^8 cfu/µg for a 10 kb plasmid.

Day 1:

Streak bacteria onto a fresh LB plate from a frozen stock. Incubate at 37°C overnight.

Day 2:

Pick a single colony from streaked plate and inoculate a 3 ml overnight pilot culture.

Day 3:

Inoculate 250 ml of SOB media (2 liter flask) with 0.5 ml of the overnight culture. Shake the 250 ml culture in a 2L Erlenmeyer flask at 20°C (or RT). Grow bacteria to OD_{600} ~0.6 (~28 hrs). The approximate doubling time is ~ 200 min for DH5α cells at 20°C. Make sure SOB, TB, and tubes for aliquots are prepared and sterile. Since RT varies, one can obtain better consistency in growing times using a temperature controlled room/incubator/water bath rather than growing at RT. Note that growth will be significantly faster at 24°C than 20°C. Keeping notes on the doubling time in your media at your standard growth temperature to simplify timing the growth. The advantage of growing at low temperature is that the cells will be between 0.2 and 1.0 OD_{600} for over 6 hrs, giving one ample time to collect cells even if one isn't exactly sure of the double time.

Day 4:

Preparation and freezing of cells. While an OD_{600} of 0.5 to 0.75 is ideal, anywhere from 0.2 to 1.0 will work.

Chill the following:

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[51] Three reasons why we don't use electroporation. First, electroporation is more complicated to perform. Electroporation requires specialized apparatus and cuvettes to provide the current zap. The cuvettes are expensive if not recycled (and this is a pain). Second, only one sample can be electroporated at a time. Most importantly from my perspective, if one adds too much salt to the cells with the DNA, the apparatus arcs and the transformation fails. This is unpredictable.

[52] We occasionally use DH10β for very large plasmids, DB3.1 or ccdB survival for growing plasmids containing ccdB. Lastly, we use BL21(λDE3) for expression of proteins. Other strains use on rare occasions include JM109 for growing F1 origin plasmids for making single strand DNA, SW102 for manipulation of BACs, EPI300 fosmid growth and for arabinose control of fosmid copy number, and DH5α *pir1*+ for plasmids with an RK6 origin.

100 ml ice-cold TB (4°C) centrifuge rotor (GSA rotor or equivalent, 4°C), 500 ml centrifuge bottles, and sterile tubes (1.5 ml Eppendorf tubes with caps that seal well at -80°C). For best results the remainder of this protocol should be done as quickly as possible and in a cold room (4°C).

When cells have reached desired OD_{600}

- Place flasks on ice for 10 minutes.
- Transfer to iced centrifuge bottle; spin 2500 x g, 10 min, 4°C.
- Gently re-suspend pellet in 80 ml ice-cold TB, pipetting and swirling on ice as necessary.
- Place on ice for 10 min.
- Spin again 2500 x g, 10 min, 4°C.
- Gently re-suspend in 20 ml ice-cold TB by swirling on ice.
-Add two 0.7 ml aliquots DMSO, while swirling on ice(to 7 % total). DMSO at RT since it is solid at 4°C.
-Place on ice, 10 min.
-Dispense into freezing tubes (~0.2 - 1 ml/tube).
-Freeze in liquid N2. Transfer to -80°C.

Notes

1) Cells can be refrozen at least once after being thawed on ice by placing back in liquid nitrogen and will continue to have high competency.

2) These cells will remain very competent for over 6 months in the -80°C, but they will keep even longer in liquid nitrogen, so if your lab uses cell only occasionally, consider only keeping only a working aliquot at -80°C.

Troubleshooting

1) The main problem we have observed is low competency we believe is due to detergent film on dishware. We have a dedicated set of two liter flasks for making competent cells that we just rinse out with distilled water after use.

2) Low quality DMSO can reduce competency greatly according to Hanahan (1983).

3) Adding magnesium to the SOB is critical.

4) Filter sterilizing units sometime contain detergent. We pre-rinse the sterilizing units with 100 ml of distilled water before filter sterilizing the TB.

Testing competent cells

The competency of cells is usually determined by transforming cells with a small amount of DNA (100 pg) of a plasmid and counting the number of colonies formed[53] (number of bacteria which took up the plasmid, survived the plating and grew up). Typically, very modest competent cell wills yield 1 x 10^6 cfu/µg, and highly competent cells will go above 1 x 10^9 cfu/µg. We typically try to use cells with a competency of over 1 x 10^8 which we can reliably make using this protocol. We usually us pBluescript or another standard vector as our test DNA. Note that transformation is dependent on the size of DNA and using a larger plasmid will yield lower 'compentency' for two reasons: 1) a 10 kb plasmid would have only 1/3 the number of molecules per ng than a ~ 3 kb pBluescript plasmid 2) larger DNA molecules transform less efficiently than smaller molecules.

[53] cfu= colony forming units

Note that one cannot test the competency of cells by adding a large amount of DNA (10-20 ng) and plating out and seeing “a lot of colonies”. Even very poorly competent cells can be successfully transformed using large amounts of DNA. Furthermore, competent cells saturate so to get an accurate idea of the competency you must use very small amounts of DNA.

Transformation

Thaw competent cells on ice[54].

Aliquot a 100 µl to pre-cooled 1.5 ml Eppendorf tubes

Add DNA solution[55] (<= 2 µl total) ,vortex briefly.

Incubate 10-40 minutes [56] on ice.

Heat shock 30-40 sec[57] at 42°C, return to ice.

Incubate 5-10 minutes[58] on ice.

Add 700 µl of SOC media[59].

Incubate 1 hr at 37° C[60] with shaking.

Plate 100 µl on an LB agar plate with appropriate selection marker. Incubate overnight. Store the remaining cells at 4°C. If more colonies are required, the remaining transformation liquid can be centrifuged at 5000 RCF for 1 minute, resuspended in 100 µl LB, and plated.

Deviating from the stated times in the transformation protocol can have negative impact on transformation efficiency (see various footnotes). Data regarding these effects come from the New England Biolabs catalog (pg 338 in the 2015-2016 catalog and found in many of their other catalogs). Our experience matches well with these comments.

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[54] This takes approximately 10 minutes. If one leaves the cells on ice a long time, the cells will settle. This may reduce competency somewhat, but I recommend just mixing gently to resuspend the cells and proceeding.

[55] One can use any volume of cells. The key is not to add too much volume to the cells. A safe guideline is not to add more than 1 µl of DNA solution per 50 µl of cells. That is the limit we stick to when adding a recently melted low melt agarose ligation recently removed from a 65°C bath.

[56] Shorter incubations will reduce transformation efficiency. Each 10 min less than 40 min reduces efficiency ~ 2 fold.

[57] We use 40 seconds for 100 µl of cells. The length of the heat shock can have dramatic effects on competency. The optimal time for heat shock depends on the type of tube, the volume of cells, and the genotype of the cells. 30-40 seconds will likely be close to the sweet spot, but only a controlled experiment can determine the optimal time.

[58] We use 10 minutes for 100 µl of cells. The length of the quenching of the heat shock can have dramatic effects on competency. The optimal time for the quenching depends on the type of tube, the volume of cells, and genotype of the cells. Only a controlled experiment can determine the optimal time (and this could be a function of the heat shock time).

[59] Use at at least 5 X competent cell volume of media. Using SOC rather than LB will increase efficiency ~2-4 fold.

[60] We perform this incubation in an Eppendorf Thermomixer at 800 rpm. The general rule is that each 15 minutes this incubation is shortened will reduce competency ~ 2 fold.

General strategies for success

DNA molecular biology often involves the multi-step protocols which are often repeated extensively. When performed carefully, the approaches are extremely reliable, but little errors can be catastrophic. Designing plasmids carefully will prevent unexpected problems. It is highly recommended that clones be created in silico before the actual experiment is performed. This is especially true of multi-step cloning projects, when failure to include an restriction site in an oligonucleotide can doom a design.

In silico plasmid sequence analysis

We use the program ApE[61] for our analysis and documentation of plasmid sequences. The program can be used to delineate features in a sequence, design primers, align sequences, search plasmids sequences with lists of oligonucleotide sequences, perform *in silico* cloning including Gateway reactions and Golden Gate cloning. Files can be saved in a standard Genbank format.

Record keeping

We keep very detailed records of how plasmids were made. We document the source of the insert and vector, the stock number and sequence of the oligonucleotides used to amplify portions of the clone, the methodology used to assemble the clone. We document what portions of the clone we confirmed by sequencing (though we do not always confirm clone identify via sequencing). We also create a sequence file (regardless of whether it is solely an expected (or predicted) or actual confirmed sequence). All this data is stored in a filemaker database. The advantage of this approach is that we can track the pedigree of our plasmid construct. If we discover an error or unexpected behavior of a clone, we can identify all clones potentially effected by the issue.

Plasmid from outside sources

It is well worth the effort of doing due diligence on any clone one receives from an outside source. Commercial sources are usually reliable, but clones from other labs are often poorly documented and their origins are often unclear. Request a sequence of the file from the source. At a minimum, perform several restriction digests and confirm the clone is of the expected structure. Sequencing key regions can save a huge amount of hassle later. For example, there are literally hundreds of different GFP derivatives. Clones with humanized codons, clones with worm codon optimizations, clones of super-folding derivatives, or with N-terminal or C-terminal polylinkers, etc. So, the label GFP in a clone does not mean much about the identity!

Precision requirements for molecular biology

The vast majority of experiments described herein do not require exacting precision. Whether you set up a restriction digest and you end up with 11 µl of total volume (such that the buffer is 0.9X instead of 1X), or if it is 9 µl (such that the buffer is 1.1X) will not matter in virtually all cases. Whether you digest 85 ng or 100 ng of DNA also will not matter. Whether you digest 45 minutes or 55 minute also probably won't matter. Whether you add 1.2 µl of 6X loading buffer or 2.5 µl of 6X loading buffer to you digest won't matter, etc., etc.

Precision can easily become a significant impediment to progress when extraordinary amounts of time are used to calculate specific volumes to pipette, and the like. Use this manual a guide, but don't hesitate to make modifications in the protocols.

[61] http://biologylabs.utah.edu/jorgensen/wayned/ape/

Recipes

Media

SOB (1 liter)

20 g Bacto tryptone
5 g Bacto yeast extract
2 ml 5M NaCl
2.5 ml of 1M KCl
Adjust volume to 1 liter and sterilize.
Supplement with 10 ml of 1M sterile $MgCl_2$, 10 ml of 1M sterile $MgSO_4$

SOC

Add 20ml of sterile 1M glucose to SOB. We make 5-10 ml aliquots and freeze them to reduce chance of contamination.

TB (1 liter)

3.35 g PIPES Na salt, final concentration: 10 mM
2.2 g $CaCl_2$ (Fisher), final concentration:15 mM
18.64g KCl (Aldrich), final concentration: 250 mM
10.9 g $MnCl_2$ (Aldrich), final concentration: 55 mM
Combine PIPES, $CaCl_2$ and KCl; pH to 6.7; add $MnCl_2$; filter sterilize.

Gel electrophoresis

50X TAE (1 liter)

242 g Tris base
57.1 mL glacial acetic acid
100 mL of 500 mM EDTA (pH 8.0)
fill up to 1 liter

5X TBE (1 liter)

54 g Tris base
27.5 g boric acid
20 mL of 500 mM EDTA (pH 8.0)
fill up to 1 liter

0.5 M EDTA pH 8.0

186.1 g EDTA disodium dihydrate in 800 ml H_20 (will not dissolve)
pH to 8.0 (requires ~ 20 g NaOH)
fill to 1 liter and autoclave

Mini-prep Solutions

P1 buffer

50 mM Tris-HCl pH 8.0
10 mM EDTA
100 µg/ml RNase A

P2 buffer

200 mM NaOH
1% SDS

Buffer N3

4.0 M guanidine hydrochloride
0.9 M potassium acetate
pH to 4.8 with acetic acid.

PE buffer

80% ethanol
10 mM Tris HCl pH 7.5

PB buffer

5 M guanidine hydrochloride

30 % ethanol

Consider adding 10 mM NaAC pH 5.2 (especially if using high pH PCR buffers like Q5 and Phusion polymerase)

TE

10 mM Tris HCl pH 8.0
0.1 mM EDTA

RNAse A (10 mg/ml)

Resuspend RNAse in 10 mM NaAc pH 5.2
Boil for 10 minutes and cool.
Store at -20°C.

2% X-gal

100 mg of X-gal in 5 ml of Di Methyl Formamide. Store at -20° C

1M IPTG

Resuspend 2.4 g of IPTG in 10 ml of water and filter sterile using a syringe and 0.2 um Filter.
Store at -20° C

Reagents

Restriction Enzymes (New England Biolabs)

T4 DNA ligase (low concentration- New England Biolabs; Cat #M0202)

Q5 polymerase (New England Biolabs, cat # M0491)

Hi-Fi DNA assembly Master Mix (New England Biolabs, Cat # E2621)

Phusion polymerase (New England Biolabs Cat # M0530)

dNTPS (New England Biolabs; Cat 3 N0046)

Gateway™ BP Clonase™ II Enzyme mix (cat # 11789-020 Invitrogen/Thermofisher)

Gateway™ LR Clonase™ II Enzyme mix (cat # 11791-020 Invitrogen/Thermofisher)

PCR purification columns (New England Biolabs Cat #T1034)

Mini-prep Columns (Monarch columns; New England Biolabs Cat # T1017)

Mini-prep Kits (QIAprep spin mini-prep kits ; Qiagen Cat # 27104)

Slides (75mm x 55 mm) Corning 2947

100 µl capillaries VWR Cat # 53432-921

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