# Protocol for cloning SEC-based repair templates using Gibson assembly and ccdB negative selection

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Overview

In our 2015 paper (Dickinson et al. *Genetics* 2015), we demonstrated the use of a Self-Excising Cassette (SEC) for drug selection to generate custom alleles (especially Fluorescent Protein fusions) in *C. elegans*. We incorporated SEC into a family of fluorescent protein (FP) tagging constructs, including the mNeonGreen (mNG) vector **pDD268** (Figure 1).
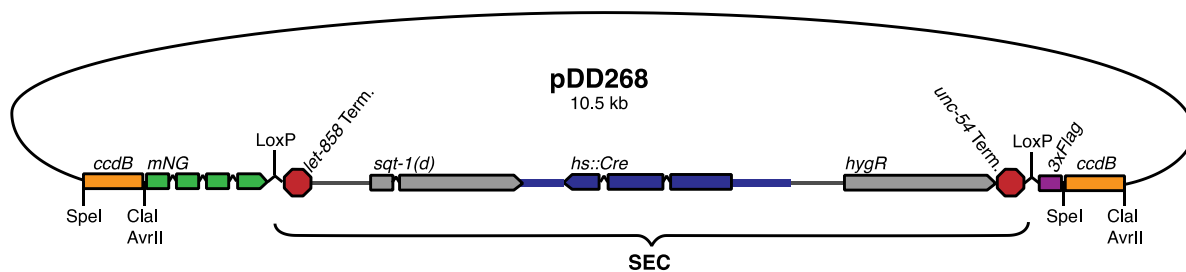
Figure 1: Map of plasmid pDD268 (mNG^SEC^3xFlag).

pDD268 and the other FP::SEC plasmids are modular constructs that can be used to tag any *C. elegans* gene. In addition to the FP, SEC and an epitope tag, these constructs include *ccdB* negative selection markers to facilitate insertion of homology arms. *ccdB* is toxic to *E. coli,* and *ccdB* negative selection is widely used as part of a Gateway cloning strategy because it eliminates parent vector background in cloning reactions. Inclusion of *ccdB* in our constructs makes cloning of 500-700 bp homology arms trivially easy. An overview of the procedure is shown in Figure 2.
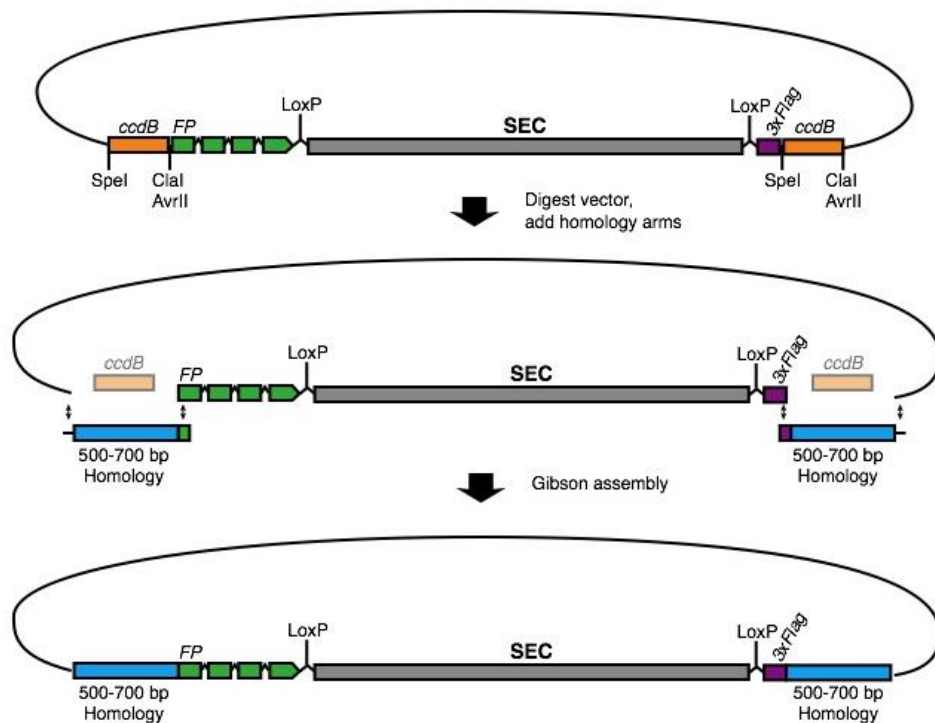
Figure 2: Cloning of homology arms into FP::SEC vectors. The FP::SEC vectors include restriction sites that cut on either side of the *ccdB* negative selection markers. PCR products that overlap the FP::SEC vector sequence are mixed with digested vector, and the homology arms are inserted in place of the *ccdB* markers via Gibson assembly.

Designing oligos for a new genome modification

Choose the Cas9 target site

1) Identify a 100-200 bp region in which the Cas9 target site should be located. We generally use a 200 bp window centered on the start codon (for N-terminal tags) or stop codon (for C-terminal tags). Using the Wormbase genome browser, retrieve the genomic coordinates for the region you want to target. In the new version of the genome browser, you can get genomic coordinates by holding your mouse over the numberline right above the annotations.

2) Submit these genomic coordinates to GuideScan (http://guidescan.com/). Enter the chromosome using roman numerals (for example, LG II would be "chrII") followed by the coordinates you want to search. Make sure you select "ce11" as the reference genome.

3) GuideScan returns both specificity and activity predictions for each sgRNA in the target region. Choose a target site that has favorable specificity, good predicted activity, and is as close as possible to the desired genome modification. In general, we try to choose target sites that have no off-targets, an activity score >40, and are within 20-30 bp of the desired modification. If it is not possible to find a target meeting all of these criteria, we suggest prioritizing specificity, followed by activity and finally distance to the desired modification.

Add the target sequence to the Cas9–sgRNA construct

1) The design tool returns target sites of the form `5'N₂₀-NGG-3'`, where N is any base. You need to insert the N_{20} sequence into the Cas9–sgRNA construct (pDD162, Addgene #47549). We use NEB's Q5 Site-Directed Mutagenesis Kit to do this. Use forward primer `5'-N₂₀GTTTTAGAGCTAGAAATAGCAAGT-3'`, where N_{20} is your 20 bp targeting sequence from the design tool, and reverse primer `5'-CAAGACATCTCGCAATAGG-3'`.

2) IMPORTANT: Do not include the PAM (NGG motif) in your primers for the Cas9-sgRNA construct. The NGG motif must be present in the target DNA, but it is not part of the sgRNA.

3) We use sequencing primer `GGTGTGAAATACCGCACAGA` to verify correct insertion of the targeting sequence.

Design primers to add homology arms to an FP::SEC vector

Figure 2 shows our strategy for cloning homology arms into FP::SEC vectors. Homology arms are generated by PCR and inserted in place of the *ccdB* negative selection markers, which are flanked by restriction sites. We chose these specific restriction sites so that no residual sequence is left behind after addition of the homology arms. *ccdB* negative selection makes this cloning strategy exceptionally robust and efficient: in a pilot experiment, we generated 8 repair templates in parallel in a single afternoon. 7/8 of these reactions yielded >80% correct clones; the remaining reaction also yielded correct clones, albeit at a lower frequency.

You need to design four primers: two for each homology arm. These primers will amplify the homology arms and add sequence overlaps for Gibson assembly to the ends of each arm. If FP::SEC insertion will not disrupt the Cas9 target site, your primers will also need to introduce silent mutations to prevent Cas9 from cutting the repair template.

Two possible pairs of restriction enzymes can be used to digest the FP::SEC vector. If AvrII and SpeI are used, the repair template will include a flexible linker between the 5' homology arm and FP (this is useful for generating C-terminal tags). If ClaI and SpeI are used, the 5' homology arm will be fused directly to the FP, with no added sequence (this is useful for N-terminal tags, or when no flexible linker is desired). Figure 3 shows sample primer designs for each situation.
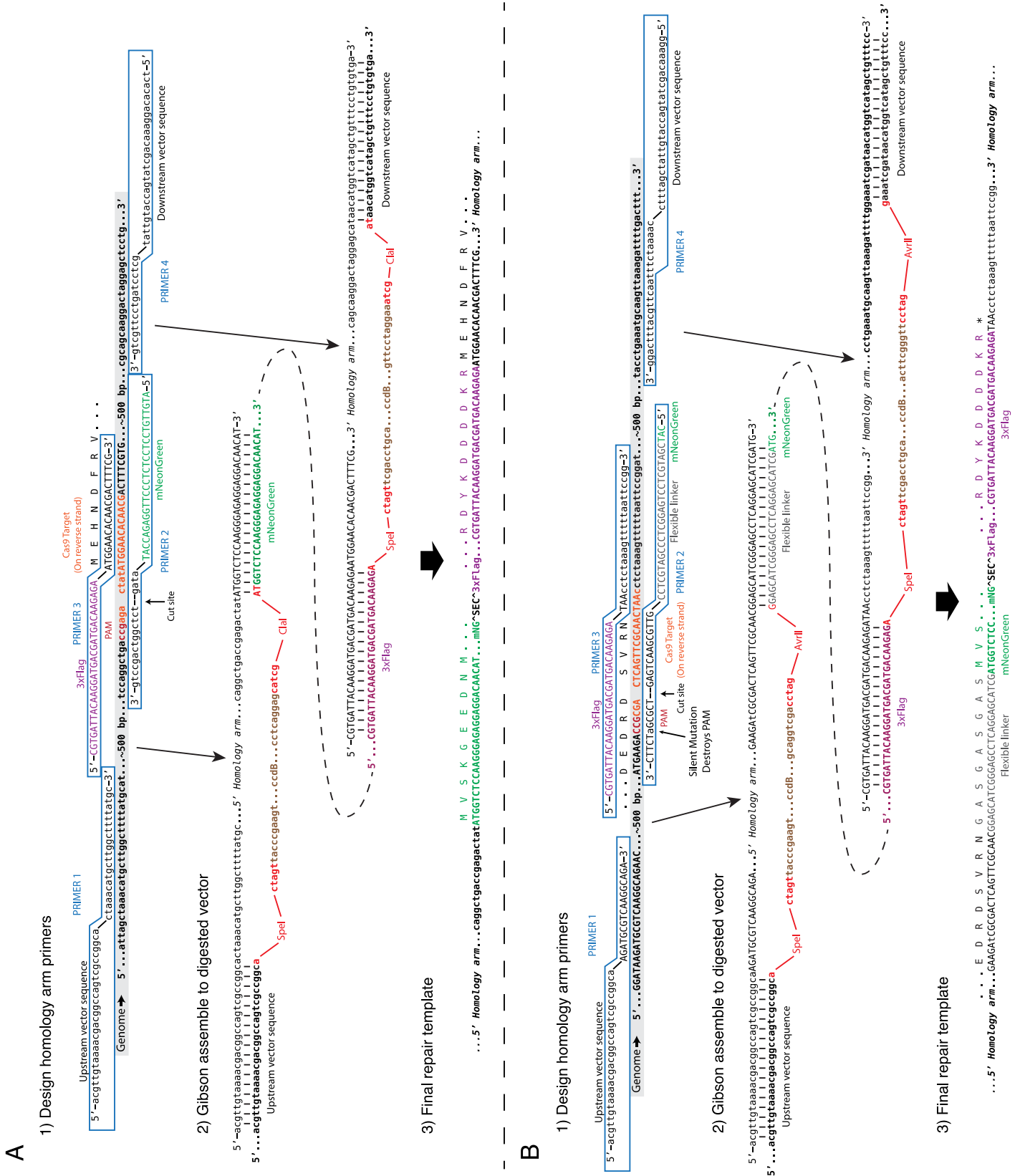
Figure 3: Primer design examples. (A) Primers for an N-terminal tag. In this example, mNG^SEC^3xFlag insertion disrupts the Cas9 target site, so no mutations are required. (B) Primers for a C-terminal tag, including the optional flexible linker that is built into the FP::SEC vectors. In this example, a silent PAM mutation is introduced to block Cas9 cleavage.

Detailed primer design instructions:

1) First, decide whether additional mutations are needed to prevent Cas9 from cutting the repair template:

    o If the insertion site is within the target sequence and within 10 bp of the PAM, no mutations are needed because the insertion will disrupt the sgRNA target.

    o Otherwise, additional mutations are made using synonymous codons so that the amino acid sequence is not altered (Figure 1).

    o If possible, the simplest and most effective approach is to mutate the PAM (NGG motif), since this motif is required for cleavage of a substrate by Cas9. (Note: while the sequence NGG is preferred, NAG can also be cleaved at some frequency, so if you mutate NGG to NAG you should also introduce a couple mutations in the target sequence to be safe.)

    o If a PAM mutation is not feasible, introduce as many mutations as possible (at least 5-6) in the target sequence.

2) Each homology arm should be 500-700 bp long. The positions of the two primers most proximal to the FP^SEC^3xFlag module (i.e., the reverse primer for the 5' homology arm and the forward primer for the 3' homology arm) are fixed by the need to insert FP^SEC^3xFlag at a specific location. The positions of the distal primers are more flexible. We design the proximal primers first based on our desired insertion site, and then use Primer-BLAST to pick the best possible distal primers.

3) Decide with FP you want to insert, and consult Table 1 for the sequence that needs to be added to the end of each primer to allow Gibson assembly.

4) Ideally, the primer length should be less than 60 bp, because longer primers are much more expensive and fail more often. If you find you need a longer primer because your Cas9 target site is far away from the insertion site, it might be more cost effective to purchase a synthetic DNA fragment (we like IDT's gBlocks) containing the homology arm instead of using PCR.

5) Before ordering primers, double check that the mNG::3xFlag will be in frame with your gene of interest.

Add homology arms to the repair template

1) Prepare the vector:

    o Digest an entire miniprep of plasmid overnight at 37°C (consult Table 1 for which construct and enzymes to use).

    o Purify the digested vector using a PCR cleanup spin column to remove the enzymes. Process the entire digested vector as one sample; do not attempt to gel purify individual bands.

- o The digested, purified vector may be stored at 4°C for at least a few months and re-used to construct multiple repair templates.

2) Prepare the homology arms:
   - o Generate two PCR products (the homology arms) using genomic DNA as the template and the primers you designed above.
   - o Mix the two PCR products and purify them together on a single PCR cleanup spin column.
3) Mix 1 µL of vector, 4 µL of homology arms and 5 µL of isothemal assembly enzyme mix (we use NEBuilder HiFi DNA assembly mix from NEB). Incubate 1h @ 50°C or as directed by the enzyme manufacturer. Transform 2 µL of the reaction to suitable competent cells.
4) Isolate DNA from 3-6 clones and sequence with M13 Forward and Reverse primers to verify correct insertion of the homology arms. This cloning procedure is efficient enough that screening clones prior to sequencing is not necessary.