Protocols for cloning SEC-based repair templates using SapTrap assembly

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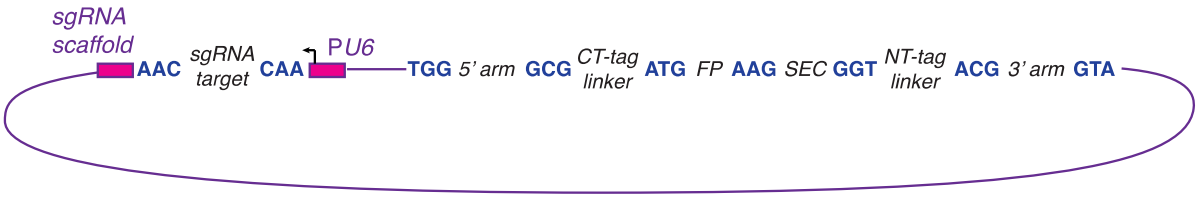
Overview

SapTrap (Schwartz and Jorgensen, 2016) is a high-throughput cloning procedure that allows modular assembly of repair templates for CRISPR/Cas9-triggered homologous recombination. Compared to the ccdB-based cloning procedure described by Dickinson et al. (2015), the SapTrap approach has several advantages:

- SapTrap is more modular: different fluorophores, epitope tags, and selection cassettes can be freely combined without the need to construct a new FP–SEC vector each time.
- SapTrap does not require the use of ccdB-containing vectors, which are somewhat prone to recombination and therefore can be difficult to grow and maintain.
- The SapTrap procedure can be used to generate a single plasmid that contains both the repair template and sgRNA – that is, all of the unique components for a genome editing procedure. This saves time by eliminating the need to clone separate Cas9–sgRNA and repair template plasmids.

In brief, SapTrap links pieces of a repair template construct together by means of unique 3-bp overlaps generated by the type II restriction enzyme SapI. Constructs generated using SapTrap have the following general form:



In a typical assembly reaction, the sgRNA target is provided as a pair of oligos, the homology arms are provided as PCR products, and the FP, SEC and linker fragments are provided by pre-existing donor vectors. These fragments are mixed together, along with the requisite enzymes, and are assembled in a single reaction. The linkers can be omitted from the final construct by designing homology arms to carry 3-bp overlaps that anneal directly to the FP or SEC.

Using SapTrap to generate repair templates for SEC selection involves two modifications to the original approach described by Schwartz and Jorgensen. First, an AAG junction is added between the FP and SEC fragments. Together, these two fragments replace the FP/unc-119(+) donors used by the Jorgensen lab. Second, it is important to ensure that a coding exon follows SEC in order to avoid nonsense-mediated decay. For this reason, we always include the NT-tag linker in our constructs, even for C-terminal tags.

Before using this protocol, you should read the papers that established these methods.

For background on the SapTrap cloning method see: Schwartz ML and Jorgensen EM (2016). SapTrap, a toolkit for high-throughput CRISPR/Cas9 gene modification in *Caenorhabditis elegans*. ***Genetics*** 202(4): 1277-88. doi: 10.1534/genetics.115.184275.

For background on SEC selection see: Dickinson DJ, Pani AM, Heppert JK, Higgins CD and Goldstein B (2015). Streamlined genome engineering with a self-excising drug selection cassette. ***Genetics*** 200(4): 1035-49. DOI: 10.1534/genetics.115.178335.

Generating new donor plasmids

Most of the time, generating new donors will not be necessary – a large variety of repair constructs can be built using donors that already exist. You only need to make a new donor if you want to incorporate a piece that does not yet exist – for example, a new fluorescent protein.

1) Determine which "slot" you need to generate a donor for – for example, fluorescent proteins go in the FP slot, selectable markers go in the SEC slot, etc.
2) In ApE, open the appropriate template file; for example, to clone a new FP donor, open the file "SapTrap FP template.gb."
3) Paste your desired sequence in place of the "NN" in the template file. Ensure that the 3-bp "overlap" sequences at the ends of the template are in the correct reading frame, if applicable.
4) Design primers to amplify the inserted sequence, using an appropriate T_M calculator. Append the *attB* and SapI sequences from the ends of the template to your primers. The primers should be designed so as to generate a PCR product with the same sequence as the template file you just created in ApE.
5) PCR amplify the insert sequence using your designed primers. Purify on a PCR cleanup spin column.
6) Set up a Gateway BP reaction by mixing 1 µL of PCR product, 1 µL of Gateway donor vector (pDONRP2rP3 for SEC donors or pDONR221 for all other donors), 2 µL of water and

1 µL of Gateway BP clonase II enzyme mix. Transform to cheap competent cells (no need to use high-efficiency cells; the BP reaction is very efficient).

7) Verify the correct donor by sequencing. You can generate the expected donor *in silico* using the Gateway recombination tool in ApE.

Designing oligos for a new repair template

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.

Design oligos carrying for the Cas9 target sequence

1) Target sites are of the form 5′N_{20}-NGG-3′, where N is any base. You need to design a pair of oligos with the N_{20} sequence flanked by 3-bp overlaps for ligation into the SapTrap donor vector. Design two 23-bp oligos: A sense oligo with the sequence 5′-TTG-N_{20}-3′ and an antisense oligo with the sequence 5′-AAC-N_{20}(antisense)-3′. For example, if your Cas9 target site is 5′-AAAAACCCCCTTTTTGGGGG-NGG-3′, then the sense oligo is 5′-TTG-AAAAACCCCCTTTTTGGGGG-3′ and the antisense oligo is 5′-AAC-CCCCCAAAAAGGGGGTTTTT-3′.

Design primers to amplify the homology arms to an FP–SEC vector

Although Schwartz et al. reported successful homologous repair using 57 bp homology arms made from annealed oligos, we have had very poor success with short homology arms in the past. Therefore, we generate 500-700 bp homology arms using PCR and add the PCR products to the SapTrap assembly reaction.

You need to design four primers: two for each homology arm. These primers will amplify the homology arms and add SapI 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.

1) First, decide whether mutations are needed to prevent Cas9 from cutting the repair template:
    - 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.
    - Otherwise, additional mutations are made using synonymous codons so that the amino acid sequence is not altered (Figure 1).
    - 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.)
    - 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 edit (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 sequence changes 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) Open the homology arm template files ("SapTrap CT 5' arm template.gb," "SapTrap NT 5' arm template.gb," "SapTrap 3' arm template.gb") in ApE. Choose the appropriate 5' arm template file based on whether you are making an N-terminal or C-terminal tag. The 3' arm template is the same in either case, because all of our repair templates include the NT-tag linker (see the note about this on page 2, above). Paste the homology arm sequence in

place of the “NN” in each template file. If necessary, edit the sequence to introduce sgRNA target site mutations.

4) Check to make sure that your designed homology arm does not contain any SapI sites. If it does, you will need to either shorten the homology arm or introduce mutations to eliminate the sites. If your homology arms have many SapI sites, or if mutating them would be problematic, an alternative is to revert to using the ccdB/Gibson based cloning strategy (Dickinson et al. 2015) instead.
5) Design primers to amplify each homology arm, including any necessary sgRNA target site mutations, and appending the SapI sites from each template to the ends of the primers. The PCR reaction should produce a product identical in sequence to your edited template file.
6) 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.

Prepare SapTrap buffers and enzyme mix

1) To prepare 8 mL of 1.25X oligo annealing buffer, combine 300 µL of 1 M HEPES pH 7.5, 1 mL of 1 M Potassium Acetate and 6.7 mL of water.
2) To prepare 40 µL of 6X SapTrap enzyme mix, combine 28 µL of SapI (NEB R0569), 6 µL T4 polynucleotide kinase (NEB M0201) and 6 µL of 400 U/µL T4 DNA ligase (NEB M0202S or M0202L). Store at -20ºC.
3) To prepare 40 µL of 6X SapTrap reaction buffer, combine 24 µL of 10X NEB T4 DNA ligase buffer, 1 µL of 1 M Potassium Acetate and 15 µL of water. Store at -20ºC.

SapTrap assembly

Your assembly reaction should include the following pieces:

- Destination vector: pDD379, pMLS256 or pMLS257
- Homology arms
- Annealed sgRNA target oligos, if your repair template will also contain the sgRNA
- FP donor of your choice
- SEC donor of your choice. Note: There are three different SEC donors that differ only in the choice of *Lox* recombination sites. pDD363 has *LoxP* sites, pDD364 has *Lox511I* sites and pDD372 has *Lox2272* sites. The three SEC donors are otherwise identical. These different

Lox variants all undergo Cre-mediated recombination with themselves, but they don't recombine with each other. This facilitates sequential modification of a strain by multiple rounds of Cas9-triggered HR with SEC selection (by using a different variant *Lox* site each time). In our lab, we've tried to stick to a convention of using *LoxP* for green FPs, *Lox2272* for red FPs and *Lox511I* for everything else – this ensures that a green strain can always be modified with a red vector and vice versa, even without advance planning.

- Linker donors of your choice. Note, to avoid nonsense-mediated decay we always include an NT-tag linker in our constructs, even for C-terminal tags.

Procedure:

1) Anneal the sgRNA target oligos:
    - Dissolve each oligo at 100 µM in water.
    - Mix 1 µL of each oligo plus 8 µL of 1.25X oligo annealing buffer in a PCR tube (10 µL total volume, final 10 µM of each oligo).
    - Anneal by heating at 95ºC for 2 min., then cooling to 4ºC at 0.1ºC/sec in a thermocycler.
    - Add 190 µL of EB (elution buffer from any DNA purification kit) to bring the annealed oligo concentration to 500 nM.
2) Prepare homology arms:
    - Generate two PCR products (the homology arms) using genomic DNA as the template and the primers you designed above.
    - Purify the products on PCR cleanup spin columns and determine the concentrations.
3) Mix 100 fmol of each donor plasmid, 100 fmol destination vector, 100 fmol each homology arm, and 300 fmol sgRNA duplex. We usually prepare donor vector stocks that are 100 nM, so 100 fmol = 1 µL. If your donor stocks and homology arms are a different concentration, adjust the volume accordingly – the molar ratio of fragments is more important than the volume. The total volume of this DNA mixture will vary depending on the concentrations and number of fragments you are assembling, but should be between 5 and 15 µL.
4) Combine 1 µL of DNA mixture from step 3, 1 µL of 6X SapTrap reaction buffer, 3 µL of water and 1 µL of 6X SapTrap enzyme mix. Incubate 2-4 hours at room temperature.
5) Transform 2 µL of the reaction to suitable competent cells (we like DH10B for cloning SEC repair templates, because this strain efficiently accepts larger plasmids). Store the remainder of the reaction at -20ºC.
6) Isolate DNA from 3-6 clones.

7) Optional: Verify correct assembly by restriction digestion. This step may not be necessary, but it doesn't take that much extra time and can save money on sequencing costs.
8) Sequence each clone with 3 primers: M13 Forward and Reverse primers to verify correct insertion of the homology arms, and with primer `5′-CATGGTCATAGCTGTTTCC-3′` to verify the correct sgRNA target sequence.

Troubleshooting

If you don't get correct colonies from your assembly, try the following:

- Double check that your homology arms don't contain SapI sites. If they do, re-design to eliminate the SapI sites.
- Re-do the assembly reaction with freshly-prepared SapTrap reaction buffer and enzyme mix. The SapI enzyme is a bit finicky, and the buffer contains ATP, so either the buffer or the enzyme mix can lose activity if not handled carefully. We have found on several occasions that just making new enzyme mix will fix a troublesome assembly.
- If you get lots of clones that look like parent vector, try restriction digestion to eliminate the destination vector background (see the Schwartz and Jorgensen paper for details). In our experience, this step is usually not necessary, but can help if the assembly is inefficient.
- Check that assembly is occurring by diluting your saved assembly reaction (from step 5, above) 1:10, and using 1 µL of the resulting solution as template for PCR with primer sets that amplify pairs of fragments. If you can amplify across a particular junction and generate a product of the correct size, this indicates that that junction is assembling correctly.
- If you are getting a lot of clones that look like they are partially assembled, it helps to reduce the complexity of the assembly reaction. The easiest way to do this is to use a 1:10 dilution of a failed assembly reaction as template for PCR with the reverse primer for the 5' homology arm plus the forward primer for the 3' homology arm. This should amplify a single band that contains both homology arms, the sgRNA and vector backbone (a total of 5 fragments). This product can then be purified and used in a new assembly reaction with the remaining pieces (FP, SEC and linker donor vectors).