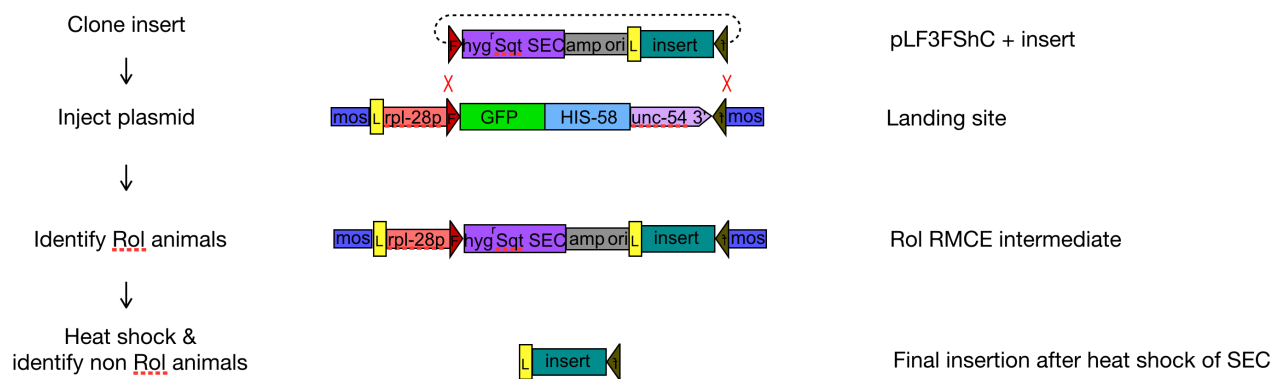


RMCE protocol

Michael Nonet, Department of Neuroscience, WUMS, St. Louis, MO 63110

Overview:

Flp recombinase mediated cassette exchange (RMCE) is a method for inserting specific DNA sequences at specific sites in the genome. The method takes advantage of both the Flp/*FRT* system and the Cre/*loxP* system to integrate plasmid derived sequences into a specific locus called a “landing site” (Figure 1). Each step of the process is monitored by visual screens. In brief, the method consists of six steps (Figure 2 & 3). First, the insert of interest is cloned into a specialized vector that contains *loxP*, *FRT* and *FRT3* sites as well as a self-excising cassette (SEC) that also expresses *sqt-1(e1350)* visible marker. Second, the plasmid is injected into a strain expressing *FLP* in the germline, and harboring a *loxP*, *FRT* and *FRT3* tagged landing site that expresses *GFP-his-58* under the control of the ubiquitous *rpl-28* promoter. Third, Rol progeny of the injected animal are identified and pooled. Fourth, the progeny of the pooled animals are screened for Rol F2 progeny. Greater than 90% of F2 Rol progeny are integration events. Fifth, homozygous integrants are identified as somatic GFP⁽⁻⁾ F3 progeny. Sixth, the SEC is excised by heat shock yielding the final integrant. In my hands, the rate of



insertion is greater than 1 per 3 injected P0 animals (see Figure S1).

Figure 1. Overview of the *C. elegans* RMCE single copy insertion method.

Detailed protocol:

I. Build your insertion construct

Insert the DNA fragment to be integrated into the genome into the pLF3FShC plasmid (Figure 4). This vector contains a small polylinker containing *SpeI*, *SbfI*, *BstEII*, *MluI*, *SphI* and *XmaI* restriction endonuclease sites. The plasmid is also compatible with *SapI*, Golden Gate and Gibson multi-insert cloning methods. A cloning manual that describes Golden Gate and Gibson assembly methods is available on the Nonet lab website (<https://sites.wustl.edu/nonetlab/manuscripts/>).

I have integrated constructs with inserts ranging from 1.5 kb to 8 kb using RMCE and I have not observed any strong size dependence of integration.

The pLF3FShC construct already has both a *let-858* and *tbb-2* 3' UTR. Thus, it is prudent to avoid using these 3' UTRs in designing a DNA insert, as direct repeats will result in instability of large plasmids.

II. Inject the DNA into a landing site strain

There are two different types of landing site strains:

1. **Split system.** Here the recombinase is provided by *bqSi711* IV, a transgene that expresses *FLP* in the germline in an operon along with *mNeonGreen* (Figure 2) that is detectable on the dissecting fluorescent microscope in late L4s, adults and early embryos. The second component is an insertion locus. Currently, the only well-characterized insertion locus is *jsTi1453* I. Other insertions can be created if one cannot use a Chr I insertion due to other genetic constraints (see below). *jsTi1453* animals express *GFP-his-58* in all nuclei and this transgene readily detected in L1s through L4s on the dissecting microscope using a high power objective, and less easily detected in adults.
2. **Combined system.** Here *FLP* is within the insertions locus and is removed by heat shock Cre-mediated excision of the SEC (Figure 3). *jsTi1493* is the most well characterized of this type of insertion locus. This transgene expresses *mNeonGreen* in the germline very similarly to *bqSi711* being detectable in L4s, adults and early embryos. It also expresses *GFP-his-58* in all nuclei at levels comparable to *jsTi1453*. The best characterized combined integration locus is *jsTi1493* IV, but *jsIs1490* IV and *jsTi1492* II are also available.

The RMCE protocol is identical for the two systems. The only difference is that if the split system is used, the new insert needs to be crossed out of the *bqSi711* background after the integration steps have been completed.

A. Inject the DNA construct

Inject young adult landing strain animals with 50 ng/μl of the construct in TE. Addition of pBluescript carrier DNA (50-100 ng/ul) may increase integration rates, but is not required. I typically inject 12 animals and place them on 4 plates.

B. Incubate injected animals at 25°C for 2.5 days.

I have also obtained integrants growing the worms at 22.5°C in one experiment. Using 25°C is faster, so that is what I recommend. Warning: 25°C is the limit of healthy growth for *C. elegans*. If your incubator is mis-calibrated, or fluctuates wildly in temperature, you may have difficulties.

C. Screen the plates for Rol animals

The Rol animals may not be as healthy as the non-Rol animals. I pool 5 to 6 Rol animals per plate and try to put animals of the same age on the same plate. I often screen the plates again 12 hours later.

D. Incubate F1 Rol plates at 25°C for 2.5 to 3 days

E. Screen F1 Rol plates for F2 Rol animals

Two types of plates appear:

- A. The vast majority are plates where there will be either no F2 Rol animals or a few F2 Rol animals (1-5). These plates need to be screened carefully to find these rare animals.
- B. A minority of plates (~ 5%-10%) will have a large number of Rol animals from 10-50 or more. These plates often are an indication that an RMCE occurred in the P0 germline or early in the development of the germline of a F1 animal. Usually, in these cases one will be able to identify homozygous insertion Rol animals as progeny that express no somatic nuclear GFP (but still express germline GFP). As *rpl-28p GFP-his-58* is brightest in L2 -L3 animals, I focus on these animals to identify animals homozygous for the insertion.

The most efficient strategy is to screen plates briefly to see if any are any B type plates. Then, one only needs to clone a few somatic-GFP⁽⁻⁾ Rol animals and one has isolated the insertion.

If no B types plates are found, one must screen the plates carefully for Rol animals. If I find more than 3 Rol animals on a plate I usually clone 2 animals to individual plates, and several others to a 3rd plate. On approximately 25% of plates that yielded insertions, only a single F2 Rol was present on the plate. Nevertheless, if the animal is clearly an F2 animal (a younger animal L2-L4) rather than a slow growing F1 roller, it is virtually certain it is an integrant. Sometimes it is unclear if an adult Rol is an F2 or an F1 animal. Some of these are indeed F2s, but others (that usually only lay only a few progeny) are smaller F1 Rol animals that have already laid many progeny. I recommend picking the adult Rol animals only if there are no plates with larval Rol animals.

F. Screen F2 Rol animals for F3 transmission

Screen for Rol animals (one should see 3/4 Rol animals or 100% Rol if it is a homozygous insertion). Clone several somatic GFP⁽⁻⁾ Rol animals (which are easiest to unambiguously score as L2-L4 animals) and grow one generation to generate a large pool of young homozygous Rol animals. If you are in a hurry you can simply pick a large number of homozygotes to heat shock, but a single het picked by mistake will ruin the heat shock.

G. Heat shock animals to remove the *sqt-1* marker cassette

Pick 15 late L1/early L2 (very small Rol animals) to each of 2-4 plates and heat shock at 30°C for 18 hrs, and return to 22.5 °C. Allow the plates to grow for 4-5 days. Other heat shock paradigms include 40 min at 37°C, 4 hr at 34°C. In my experience 30°C is most reliable.

H. Screen heat shock plates for non-Rol animals

This step is particularly random. Sometimes, it is quite difficult to get an excision and it takes screening greater than 5 plates of a dozen heat shocked animals to isolate a non-Rol animal, while other times it even occurs spontaneously without heat shock.

III. Molecularly confirm the insertion structure

In some cases, one can be relatively confident an insertion is correct. One may have very specific expectations about the expression pattern and expression levels due to prior experience with a gene.

However, other times it is difficult to know if the insertion is correct from a phenotypic characterization of the strain. I typically characterize the transgene by amplifying across the entire insertion locus using a long range PCR protocol, and then digesting the PCR product with several enzymes to confirm the DNA is of expected structure. To date, I have not observed any incorrect or incomplete insertions into *jsTi1453* or *jsTi1493*, so this step is not essential, but still prudent to perform.

Preparation of genomic DNA

Wash off a recently starved standard plate using 1 ml of H₂O and place in a 1.5ml microfuge tube. Spin to pellet worms at 3,000 g for 10 seconds, and remove all but 50 µl of the H₂O. Freeze the worms, then thaw and add 150 µl of lysis buffer (10mM Tris, 2mM EDTA, 1% SDS, 50 µg/ml proteinase K). Incubate at 60°C for 1 hr, vortexing the tube for a few seconds every 20 min. Cool to 37°C, add 1/2 µl of 10 mg/ml RNase A and incubate for 20 min. Add 200 µl of 3 M Guanidine HCl, 3.75 M NH₄Ac, pH 6 and mix. The solution will become cloudy. Add 200 µl of ethanol and mix. The solution will clear. Load the DNA onto a Qiagen QIAquick spin column (usually used for DNA fragment purification). Spin 30 seconds in a microfuge at full speed. Add 750 µl of PE buffer (80% ethanol, 10mM Tris pH 7.5) and spin 5 seconds. Remove wash solution. Repeat the PE wash. Spin 1 minute to remove traces of the wash solution. Add 100 µl of TE (10 mM Tris 8.0, 0.1 mM EDTA). Incubate 5 minutes at room temperature and then spin 1 minute in a microfuge to collect DNA. Typical yield is about 1 µg of genomic DNA.

PCR using oligonucleotide using a long range PCR

Perform a 15 µl PCR reaction using NEB Long Amp Polymerase. This polymerase is extremely effective at producing high yields of long PCR products. However, the error rate is very high, so don't be tempted to use the enzyme for cloning. A 15 µl reaction will usually yield ~ 1.5 µg of PCR product using the following amplification.

3 µl of 5X Long Amp Buffer
2 µl of 2.5 mM dNTPs
1 µl of 2.5 µM oligo 1
1 µl of 2.5 µM oligo 2
7.375 µl of H₂O
0.5 µl of genomic DNA (~5 ng)
0.125 µl of Long Amp Polymerase

PCR conditions:

0:30 @ 95°C, 30X (0:10 @ 94°C, 0:50 @ 60°C, 1 min/kb @ 65°C), 10:00 @ 65°C.

Oligonucleotides:

jsTi1453 js1453f caaaagccctactatcaacgc & js1453r catcccattcacggcacaac
jsTi1493 js1493f tcagtccctcgagcagtcagg & js1493r ttcgcaaaccgtctgcgtctc

Purify the PCR product using a standard DNA clean up procedure such as a NEB Monarch[®] column PCR & DNA fragment protocol. Restriction digest the product with appropriate enzymes to assess the DNA insert structure.

IV. Creating novel insertion landing sites.

Two approaches can be used to create additional landing sites. First, one can use the miniMos vectors to create new randomly positioned insertions. Second, I have created two SapI CT-NT golden gate vectors that contain the split landing site and the combined landing site. These are intended to be used as the central insert in a three part SapI Golden Gate cloning that also contains a 5' arm and 3' arm homology ends. The resulting vector can then be used in conjunction with either a MosSCI or CRISPR directed transgene insertion method to create a novel landing site.

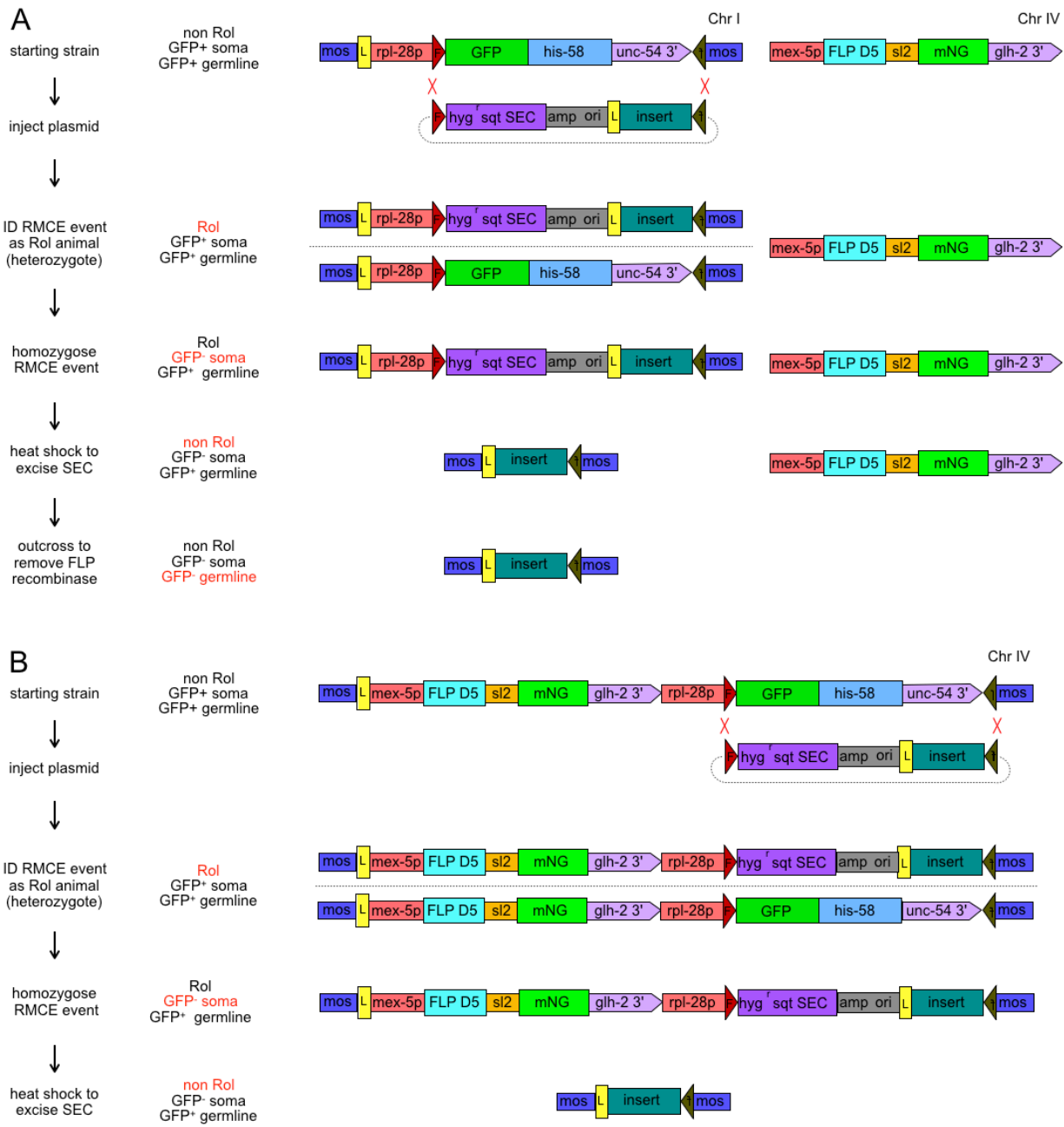


Figure 2 Outline of the RMCE method

A) two component approach and B) single component approach

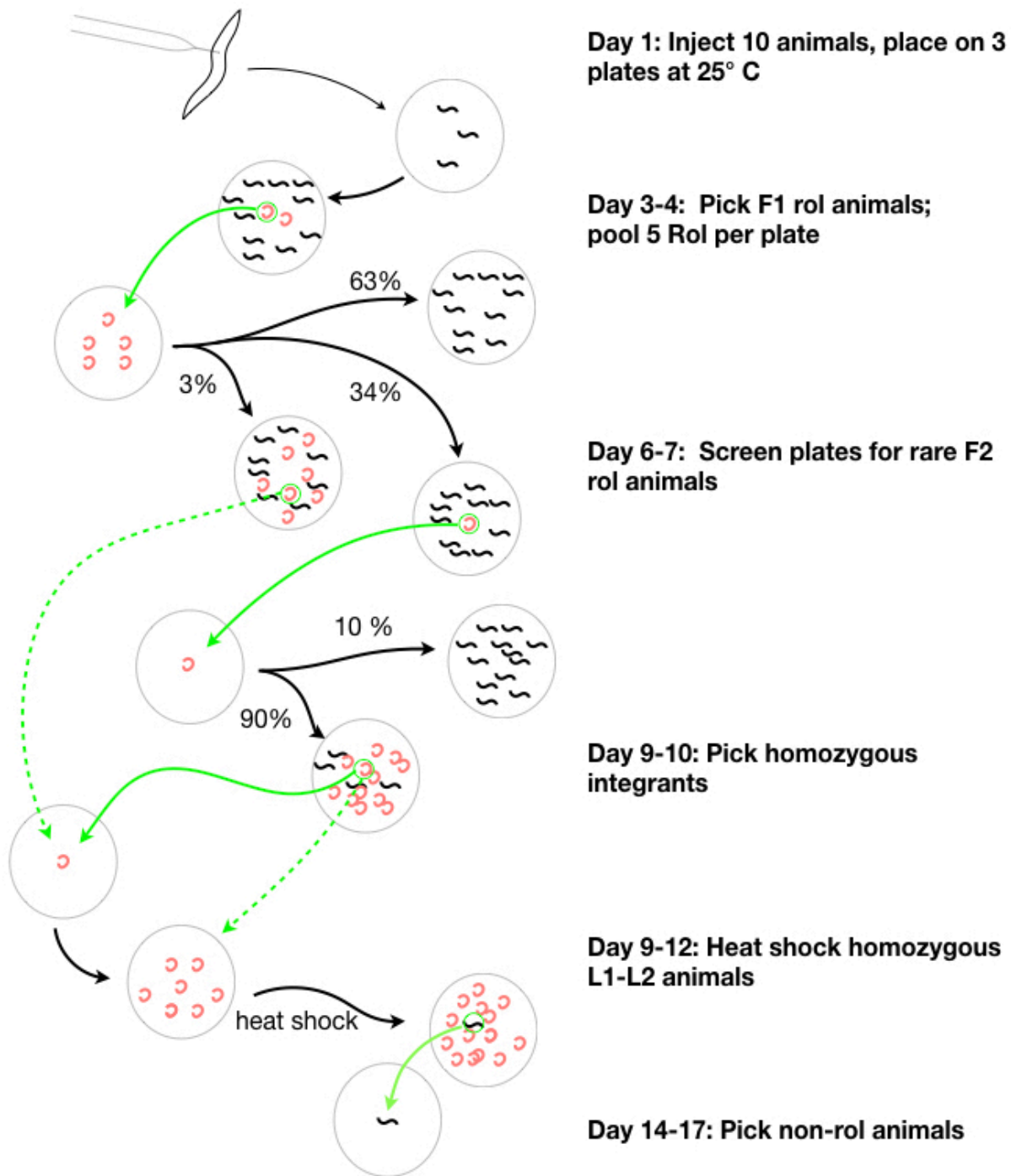


Figure 3. Timeline of RMCE integration method.

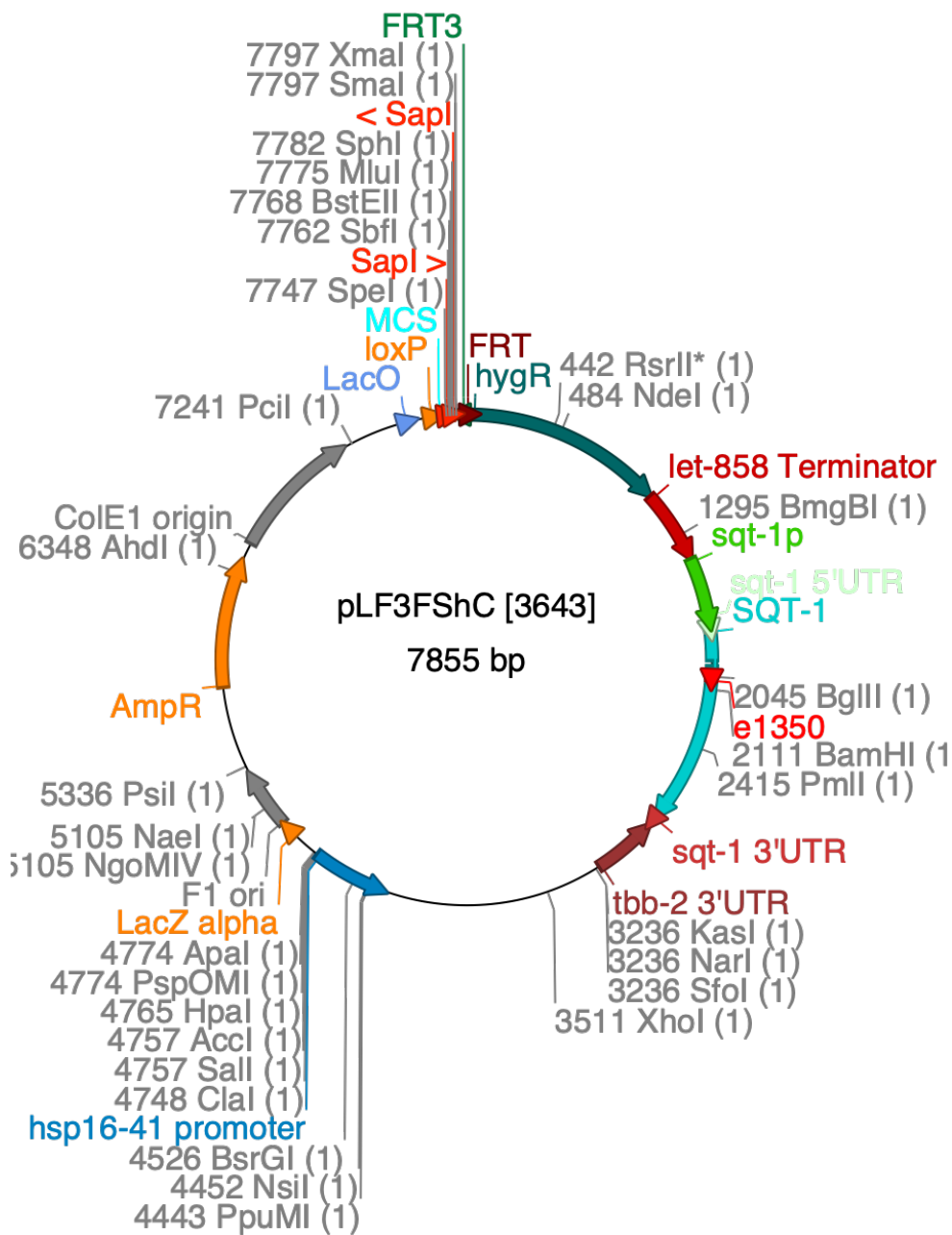


Figure 3. Restriction map of pLF3FShC