RMCE protocol

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(DRAFT - PLEASE DO NOT DISTRIBUTE)

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). In the current version of the method, 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 D5 in the germline, and harboring a loxP FRT FRT3 tagged landing site that expresses GFP-HIS-58 under the control of the ubiquitous *rpl-28* promoter. Third, RoI progeny of the injected animal are identified and pooled. Fourth, the progeny of the pooled animals are screened for RoI F2 progeny. Greater than 90% of F2 RoI 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 2.8 injected P0 animals[1].

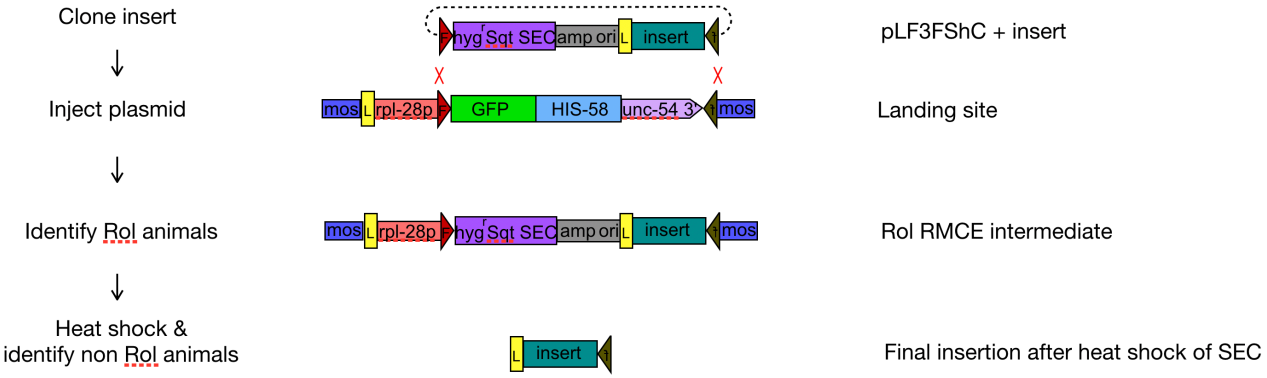

Figure 1. Overview of the *C. elegans* RMCE single copy insertion method.

Detailed protocol:

I. Build your insertion construct

Insert the DNA fragment to be integrated into the genome into the pLF3FShC plasmid. This vector contains a modest 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. My lab's experience is that one can easily build 20 kb plasmids using DH5α the *E. coli* host. For reference, the pLF3FShC vector is 8 kb.

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[1] As of Dec 2019, 30 experiments averaging 8.8 injected animals per experiment yielded an average of 3.1 insertions per experiment. 28 of 30 injections experiments yielded at least one insertion. This frequency under represents the true frequency since 1) I pool animals and likely undercounted events because where two independent events occurred, and 2) I tested lower and upper limits for optimal DNA concentration for the injections.

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 D5 in the germline in an operon along with mNeonGreen (Figure 2). The mNeonGreen expressed by this transgene 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 is 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 remove 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 expressed 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. I typically inject 12 animals[2] 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 you incubator is mis-calibrated, or fluctuates wildly in temperature, you may have unexpected issues.

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 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.

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[2] Injection techniques vary. In my hands, a typical injection of 12 N2 young adults with (50 ng/µl of pRF4 [*rol-6(e1005)*] + 100 ng/µl of pBluescript) will yield an average of >15 rol/ per injected animal with a >80% survival rate of injected animals. I also typically score the injections as “good” where clear gonad flow of the injected liquid is observed or “questionable” and regularly obtain 75% “good” injections. Injections of pLF3FShC plasmids into the RMCE strains yield less Rol animals, typically averaging approximate 6 Rol progeny per injected animal (1594 F1 Rol from 255 injected animals). Note that there is significant variance in injections. See Supplementary Table 1 for examples.

B. A minority of plates[3] 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 the rpl-28p GFP-HIS-58 is best detected 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 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. Approximately 25% fraction of plates that yielded insertions[4], only a single F2 Rol is present on the plate. Nevertheless, if the animal is clearly an F2 animal (a younger animal L2-L4) rather that a slow growing F1 roller, it is virtually certain it is an integrant[5]. Sometimes it is unclear if an adult Rol is an F2 or an F1 animal[6]. 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 general a large pool of young homozygous Rol animals. If you are in a hurry see footnote[7].

G. Heat shock animals to remove the *sqt-1* marker cassette[8]

Pick 12 L2 (very small Rol animals) to each of 2-4 plates and heat shock at 37°C for 40 minutes, and return to 22.5 °C. Allow the plates to grow for 4-5 days.

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. I am not sure why this is the case.

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

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[3] 8 of 92 plates that yielded insertions in 30 injection experiments

[4] 26 of 92 plates that yielded insertions in 30 injection experiments

[5] 92 of 103 F2 Rol plates transmitted. Of the 11 that did not transmit most were likely to have been F1 progeny rather than F2 progeny.

[6] Transferring the Rol progeny can eliminate this issue, but I find it is not worth the effort.

[7] If the F2 animal laid a large brood and one screens the plate early enough while many animals are larvae one call identify enough homozygous animals to heat shock without amplifying the homozygous mutant population. However, contamination with a single heterozygote will foil the attempt. Furthermore, spontaneous excisions sometime occur. See footnote 7.

[8] It is worth screening the progeny of the homozygous F3 Rol plate for rare non-Rol animals as the SEC occasionally excises without heat shock. Check that the non-Rol animal is somatic GFP$^-$ to confirm the non-Rol is a real excision event.

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, we have not observed any incorrect or incomplete insertions into j*sTi1453* or *jsTi1493,* so this step is unnecessary, but still prudent to perform.

Preparation of genomic DNA

Wash off a recently starved standard plate using 1 ml of H_20 and place in an 1.5ml microfuge tube. Spin to pellet worms at 3,000 g for 10 seconds, and remove all but 50 µl of the H_20. 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.[9] 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 a producing large 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 uM oligo 1
1 µl of 2.5 uM oligo 2
7.5 µl of H_2O
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 +0:05/cycle[10]), 10:00 @ 65°C.

Oligonucleotides:
jsTi1453 js1453f caaaagccctactatcaacgc & js1453r catcccattcacggcacaac
jsTi1493 js1493f tcagtcctcgagcagtcagg & 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 I used to create the currently available insertions and create new randomly positioned

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[9] Alternatively one can extract with 200 µl of phenol/chloroform, extract with 200 µl of chloroform, then ethanol precipitate by adding 20 µl 3M Na acetate pH 5.2, add 450 µl of ethanol and spinning 15 minutes in a microfuge. After removing the supernatant, wash the pellet with 400 µl of 70% ethanol, and resuspend DNA in 100 µl of TE.

[10] MJ Research PTC100 thermocycler using the EXTEND function to increase the extension time each cycle. I doubt this is really needed.

insertions. Second, I have created two SapI CT-NT golden gate vectors[11] 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.

Please contact me to provide feedback about your experience with RMCE.

Figure 2: Split system work flow:

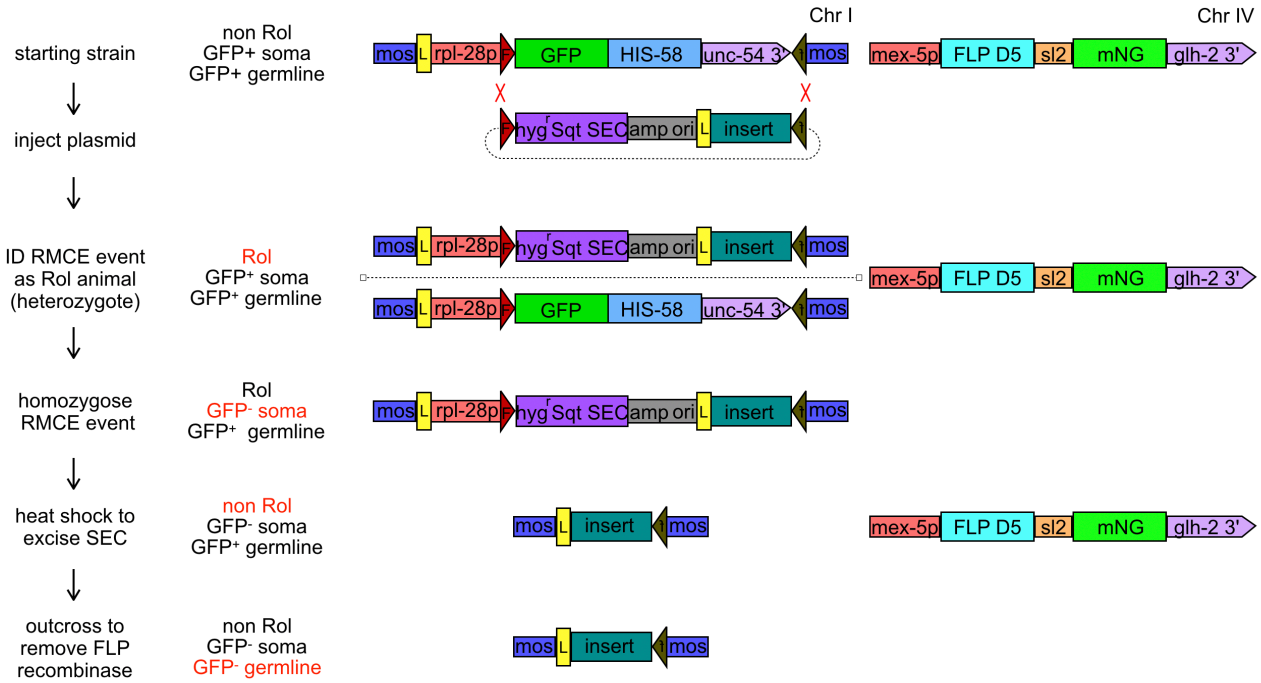
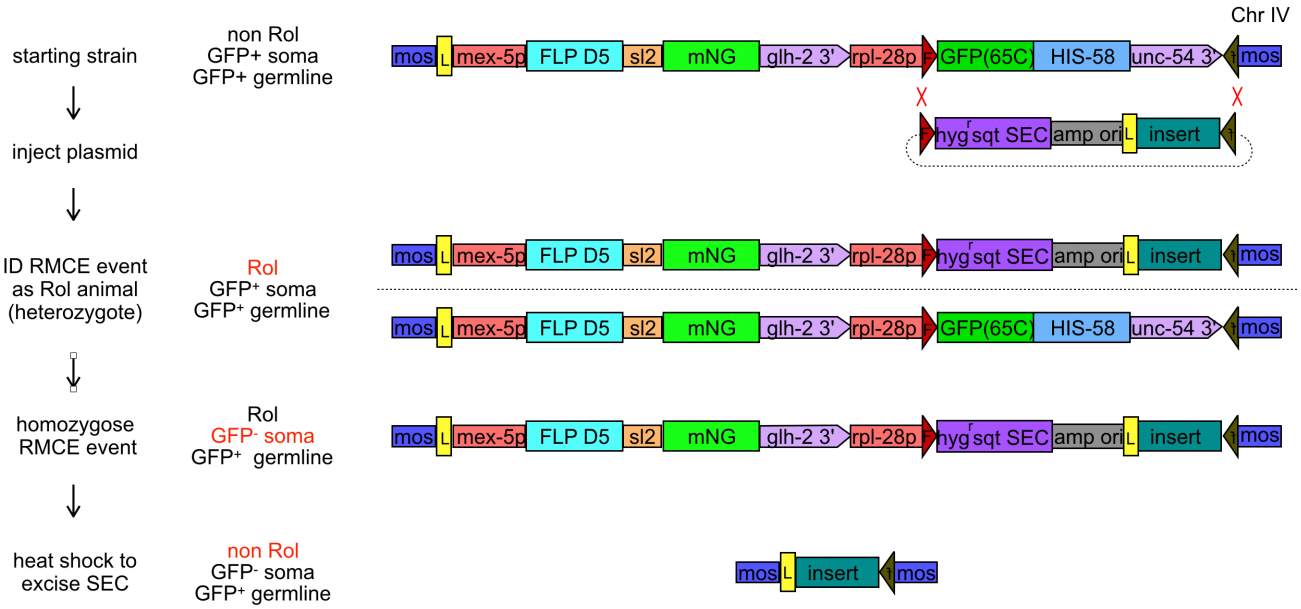


Figure 3: Combined system work flow:



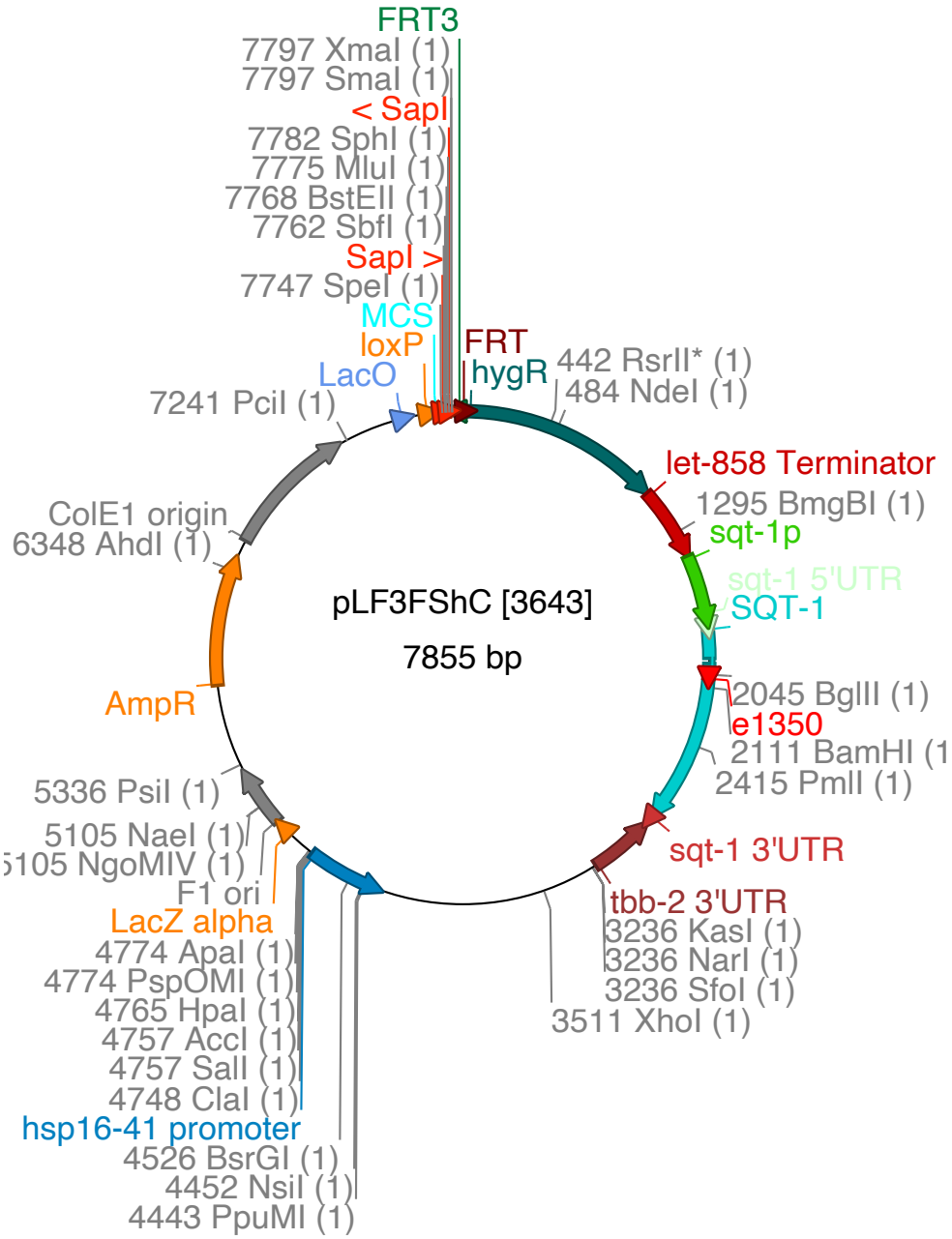


Figure 4. Restriction map of pLF3FShC