

PhiC31 recombination mediated cassette exchange (RMCE) protocol

Overview:

PhiC31 recombination mediated cassette exchange (RMCE) is a method to insert a single copy of DNA into the genome of a pre-engineered phiC31 landing pad carrying strain, e.g., BRC0566. Two "inward" facing phiC31 attP sequences (50 bp) along with phiC31 gene and a *myo-2p::GFP* marker have been integrated at the *tTi5605* site on LG II by MosSCI. Using a donor plasmid carrying "inward" facing phiC31 attB sites (38 or 40 bp), one can easily insert DNA fragments into BRC0566. Two attR sequences will be generated after the integration at both junctions that can be molecularly validate by PCR and Sanger sequencing (**Fig. 1A**). BRC0566 displays the Unc phenotype, so a *unc-119* rescue fragment has been included into the donor plasmid. First, obtain the attP landing pad strain BRC0566 from CGC and maintain at 20°C. Second, insert the DNA fragment to be integrated into the genome into the donor plasmid. Third, microinject the construct with a co-injection marker plasmid. Fourth, screen for non-Unc progeny and subculture them until the extrachromosomal array is lost. Fifth, validate the right insertion by PCR, sequencing, and/or Southern blots. Finally, one or more outcrosses to wild-type is recommended (**Fig. 1B**).

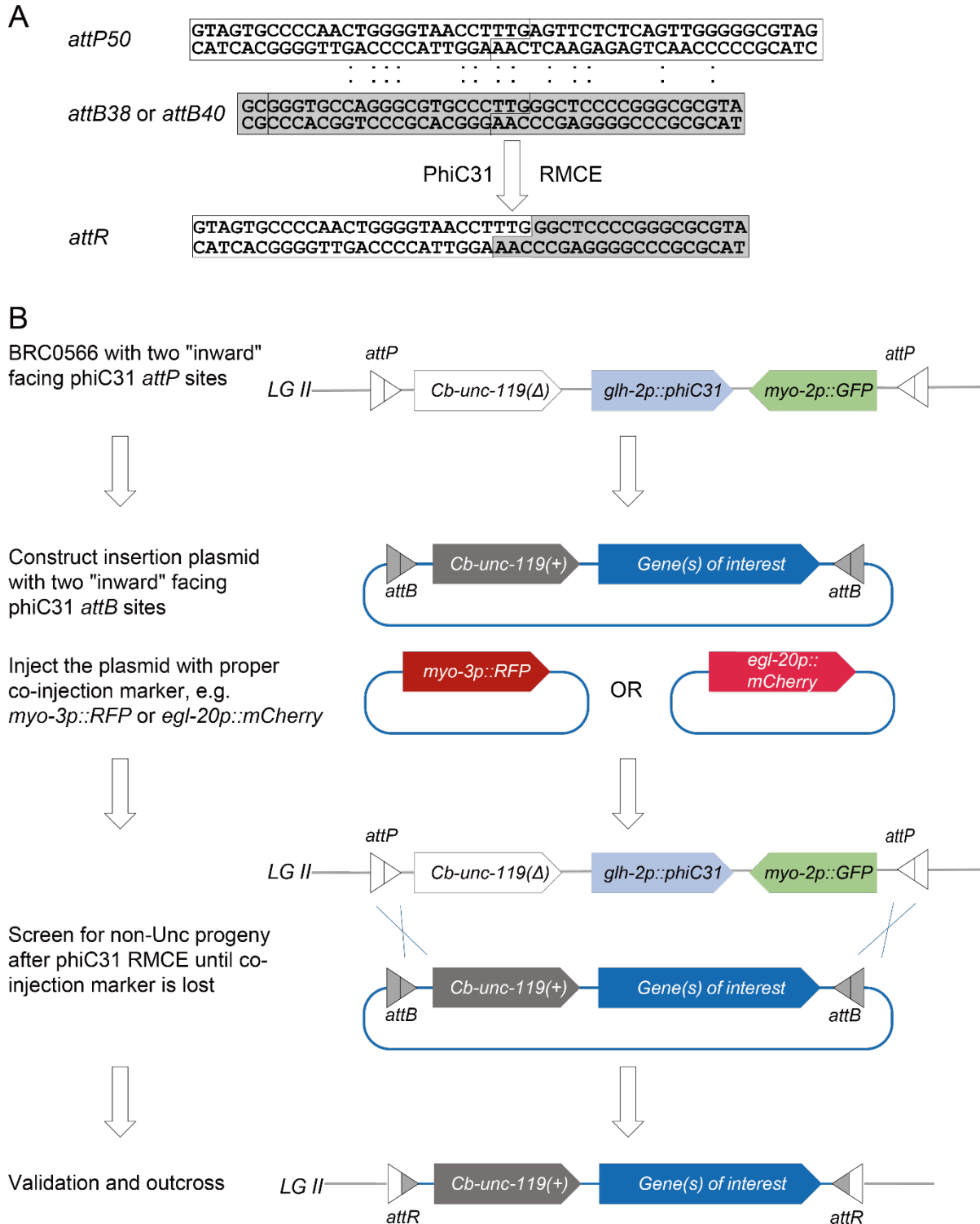


Figure 1. Overview of the *C. elegans* phiC31 RMCE. (A) Schematic of phiC31 *attB*, *attP* and *attR* sequences and the recombination site. (B) The workflow of the *C. elegans* phiC31 RMCE.

Detailed protocol:

I. Obtain docking site strain from CGC and maintain

Obtain docking site strain BRC0566 from CGC and maintain at 20°C. This strain is Unc-119 and grows slowly. BRC0566 carries *myo-2p::GFP*, but this is very faint. We have the best success seeing the GFP in the L2 or L3 stages. We seem to have poorer injection success with BRC0566 maintained at room temperature.

II. Build your insertion constructs

Two convenient plasmids containing two "inward" facing phiC31 *attB* sites for RMCE are described in this protocol: 1) pBRC_double_attB_GFP_donor (Addgene #169692) that also carries a *sur-5p::GFP* marker, and 2) pCG150_double_phiC31_attB (Addgene #169693), which carries no fluorescent reporter (**Fig. 2**). Both can accept insertions through Invitrogen Gateway cloning or by standard cloning. Insert the DNA fragment to be integrated into the genome into the pBRC_double_attB_GFP_donor plasmid using the attR1 and attR2 Gateway attachment sites or *Sall/DraIII* and for pCG150_double_phiC31_attB using the attR3 and attR4 Gateway attachment sites or *SbfI/ApaI* or *SbfI/XhoI*.

Very large constructs (>15 kb) are better cloned into low copy vectors, such as fosmids. Alternatively, fosmids and BACs can be retrofitted with *attB* sites through recombineering.

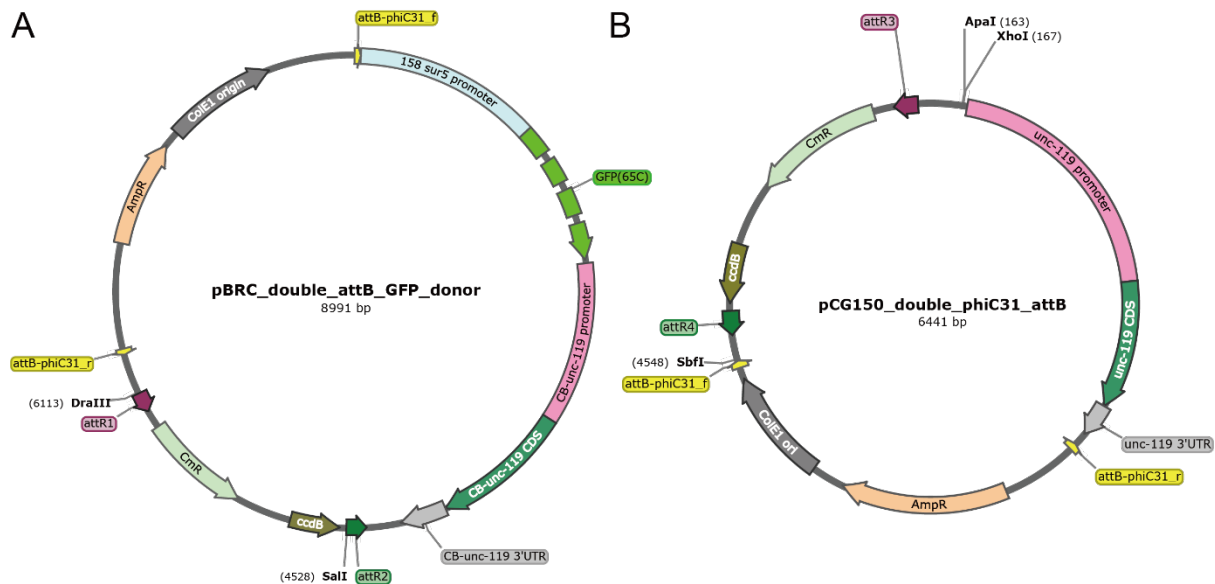


Figure 2. Maps of two phiC31 *attB* donor plasmids. (A) pBRC_double_attB_GFP_donor (Addgene #169692). (B) pCG150_double_phiC31_attB (Addgene #169693).

III. Inject the insertion construct into the BRC0566 strain with phiC31 double-*attP* landing sites

A. Inject the DNA construct.

1. Day 0. Inject the donor plasmid construct into BRC0566 using standard microinjection protocols. We typically use between 25-100 ng/μl of donor plasmid and 8-50 ng/μl of a co-injection marker. Our currently approach is to attempt to inject 10-20 P0's. After injection we usually put 5 P0's per plate. We may put less per plate if we feel there are many "well-injected" P0's (**Fig. 3**).
2. Day 1 and Day 2. Transfer P0's to new plates to help synchronize the F1 progeny for easier screening.

B. Screen the plates for non-Unc animals and integrants

1. Days 2-4. Screen for non-Unc-119 F1's. For integrated transgenes <15 kb, we single out 20-40 individuals. For larger transgenes, we single out all non-Unc F1's. We think a small percent (<25%) of integration events occur early to yield F1's heterozygous for the transgene while most integration events occur in the F1 germline to yield heterozygous F2's.
2. Day 5 and onward. Keep plates that segregate non-Unc F2. Some F1's are putative insertion heterozygotes and yield approximately 75% non-Unc F2. For these we single out 2-6 F2's, depending on the work load, to try and obtain insertion homozygous F2's. These may simultaneously still carry an extrachromosomal array which needs to be lost. This can be determined based on continued segregation of the co-injection marker. Single copy insertion of GFP or other markers are often very faint. Some F1's will segregate few non-Unc F2's, presumably because the F1 has no integration but was carrying an extrachromosomal array. However, it seems integration can happen in the F1 germline, so some F2's will be integration heterozygotes. Depending on the number in the first class, we will also pick 2-6 non-Unc F2's.
3. Continue picking in the F3 and future generations until a homozygous insertion line is established that also no longer carries an extrachromosomal array. We usually assess this by 100% non-Unc progeny, weak or faint fluorescence of the marker within the transgene (if present, e.g., *sur-5p::GFP*), and lack of the co-injection marker fluorescence (e.g., *myo-3p::RFP* or *egl-20p::mCherry*). Conversely, if the fluorescent marker(s) appear bright, then either the extrachromosomal array is still present or the Is array is complex, such as with multiple copies and including the co-injection marker. Additionally, there should be loss of the *myo-2p::GFP*.

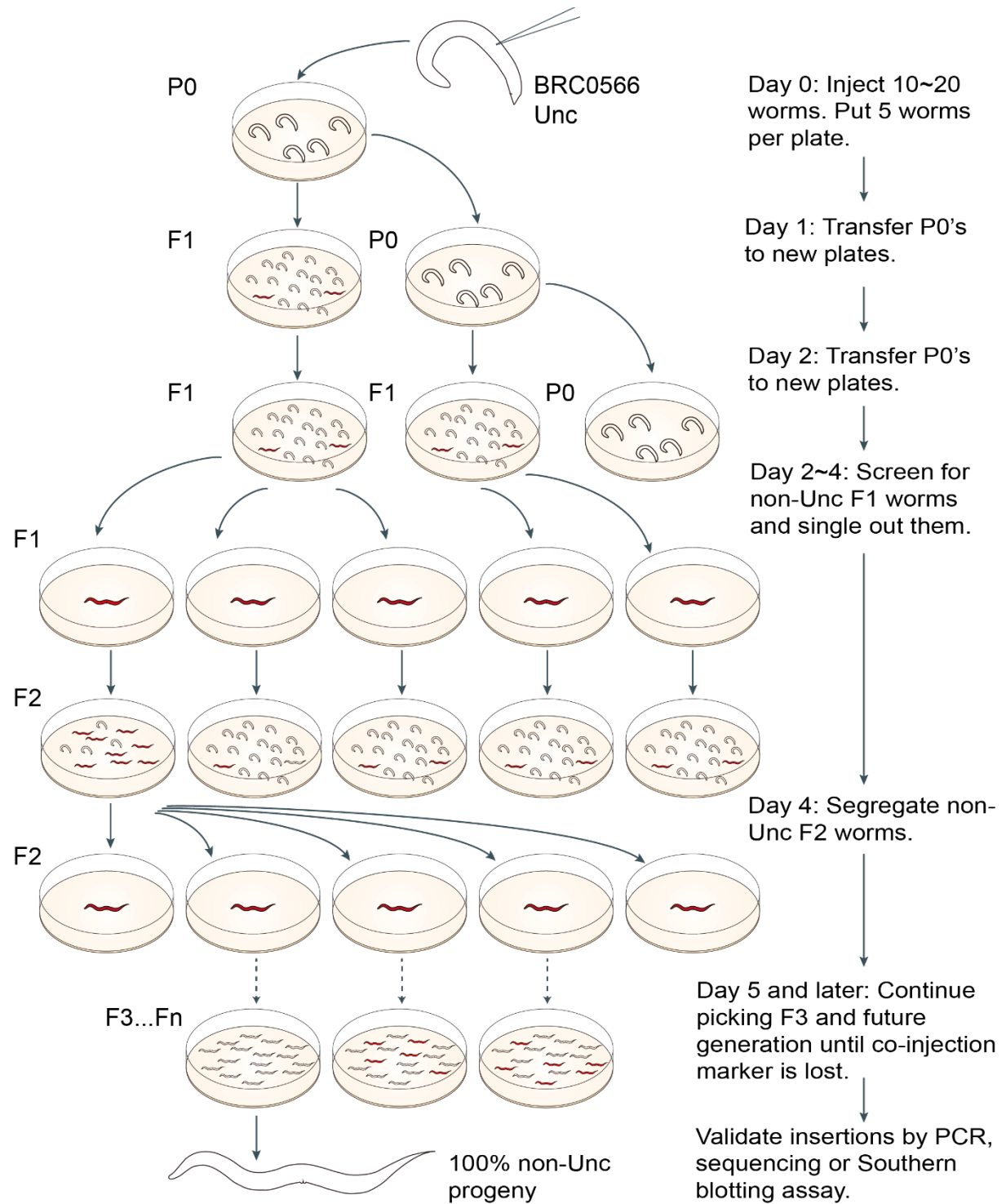


Figure 3. Time line of phiC31 integration method.

IV. Validate insertions

- A. We first use PCR with proper primers to check for junction formation (i.e., *attR* sites). Note that the current docking site can integrate the transgenes in two orientations. This can be done via single worm PCR or with DNA isolated from plates.
- B. We keep only strains with left and right positive PCR assays for the junctions that are both consistent with same orientation.
- C. If putatively correct, then there are several options:
 1. We then check for the loss of *phiC31* by PCR.
 2. If the insertion is ~10kb or shorter, we will attempt long PCR across the whole insert. Note this requires high quality DNA, which we typically obtain with the Qiagen Genomic-tips kit.
 3. Sometimes an aberrant short PCR product is seen that is the size of skipping the insert. We presume this is because the *attB/P/L/R* sites can also pair with each other. The attachment sites also form hairpins which may make PCR harder than normal. The short circuited PCR product can sometimes be brighter than proper long PCR product (see Fig. 1D and Sup. Fig. S5E for examples).
 4. For longer insertions, other validation methods can be considered including Southern analysis or Oxford Nanopore sequencing. For the current study, we sequenced to >60x depth, but this is more than necessary. Although we haven't tested with smaller subsets, ~10x is probably sufficient (corresponding to ~1 Gbp of sequence).
 5. Outcross strain. As with all genetic manipulations, it may be prudent to outcross the insertion strain.