

# Chapter 2

## Genome Manipulations with Bacterial Recombineering and Site-Specific Integration in *Drosophila*

Yi Zhang, William Schreiner, and Yikang S. Rong

### Abstract

Gene targeting is a vital tool for modern biology. The ability to efficiently and repeatedly target the same locus is made more efficient by the site-specific integrase mediated repeated targeting (SIRT) method, which combines homologous recombination, site-specific integration, and bacterial recombineering to conduct targeted modifications of individual loci. Here we describe the recombineering designs and procedures for the introduction of epitope tags, in-frame deletion mutations, and point mutations into plasmids that can later be used for SIRT.

**Key words** Bacterial recombineering, Gene targeting, *Drosophila*, Site-specific recombination, Genome engineering

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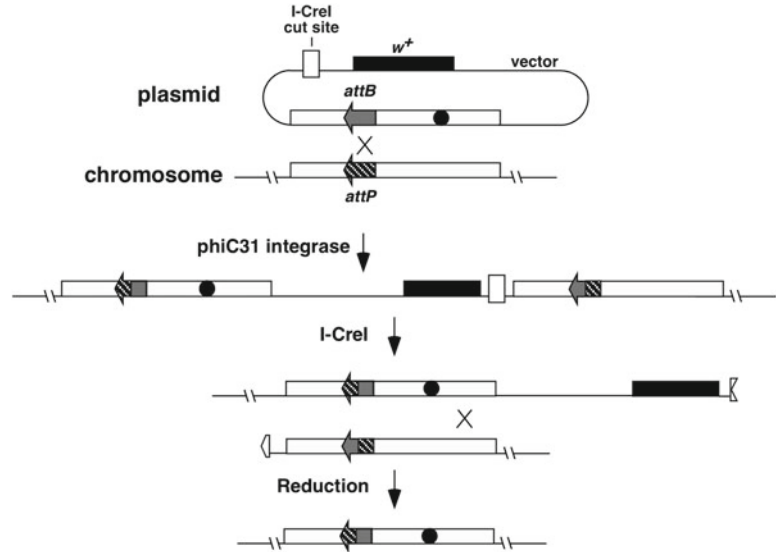
### 1 Introduction

Gene targeting by homologous recombination revolutionized the study of gene function by enabling genetic manipulations of endogenous loci in vivo. By combining homologous gene targeting and site-specific recombination, we recently developed the “Site-specific Integrase mediated Repeated Targeting” (SIRT) method for *Drosophila melanogaster*, which facilitates repeated rounds of targeted manipulation of a single locus [1]. In SIRT, homologous recombination is used to place an *attP* attachment site of the phage phiC31 integrase in the vicinity of the target locus. All subsequent modifications to the same gene are introduced as plasmids carrying the modifications and the *attB* attachment site. These plasmids are directly injected into *attP*-containing embryos expressing the phiC31 integrase. The integrase mediates an exchange between the two *att* sites, which results in plasmid integration precisely at the chromosomal *attP* site. Figure 1 provides

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Yi Zhang and William Schreiner contributed equally to this manuscript.

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**Fig. 1** SIRT. The *top diagram* depicts *phiC31*-mediated site-specific integration. An *attP* attachment site is first targeted to a locus of interest on the chromosome by traditional ends in gene targeting [9]. The *open rectangular box* represents the locus of interest. A plasmid carrying the desired modification (*filled circle*), an *attB* attachment site, a cut site of the I-CreI endonuclease, and a *white(w<sup>+</sup>)* marker gene (*filled rectangular box*), is introduced into *attP*-containing flies that express *phiC31* integrase. Plasmid integration into the chromosome, which is mediated by an exchange (“X”) between the *att* sites, results in the duplication of the target locus and the integration of the *w<sup>+</sup>* marker that gives rise to eye pigmentation. The *bottom diagram* depicts I-CreI-mediated reduction of the target duplication. I-CreI expression is induced to generate a DNA break at its cut site. This break induces recombination between the two target copies. If recombination occurs at the position denoted by the “X”, the reduction product will harbor the desired modification. Flies with successful reduction events will be white-eyed due to the loss of the *w<sup>+</sup>* marker

a detail description of the steps in SIRT. We have successfully used SIRT to modify multiple genes important for telomere maintenance in *Drosophila* (e.g., 1, 2). These modifications include deletion of a locus, small in-frame deletions, point mutations, and insertions of epitope tags.

To construct plasmids for SIRT, we extensively utilize the bacterial recombineering technology, which is based on recombination induced by the expression of the RED system from phage lambda [3]. In this chapter, using the *verrocchio* (*ver*, 4), *hiphop* [5], and *caravaggio* (*cav*, 6) loci as specific examples, we describe recombineering designs and procedures for introducing epitope tags, in-frame deletion mutations, and point mutations. Once the final plasmid is generated, it can be introduced into flies by SIRT protocols previously described [1, 7].

Although we mainly use SIRT for gene disruption, the same practice can be used to achieve gene correction. Even though our SIRT method was specifically developed for *Drosophila*, all of its components are functional in other eukaryotes making it easily adaptable in other model organisms.

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## 2 Materials

### 2.1 PCR Primers

Primers used for recombineering are generally longer than 70 bp as each primer would minimally include 50 bp homologous sequence to the target gene and 20 bp homologous sequence to an antibiotic resistant gene. In addition, restriction enzyme cut sites are often included in the primers to facilitate excision of the antibiotic marker. For specific design of primers, *see* discussion in the Subheading 3. Purification of the primers is not necessary.

### 2.2 Bacterial Strains and Culturing Materials

1. A strain that is competent for recombineering. We used the SW102 strain [8]. For other available strains, *see* <http://redrecombineering.ncifcrf.gov/>.
2. Standard Bacterial cloning strains such as DH5 $\alpha$  and DH10 $\beta$ .
3. LB liquid medium.
4. SOC medium.
5. Antibiotics.
6. Bacterial Culture Tubes.
7. Bacterial Culture flasks.
8. Bacterial electroporator.
9. Electroporation cuvettes.
10. Shaking water bath.

### 2.3 Enzymes and Buffers

1. DNA polymerase for PCR with proofreading activities.
2. Restriction enzymes and buffers.
3. DNA ligase and buffer.

### 2.4 Molecular Biology Kits

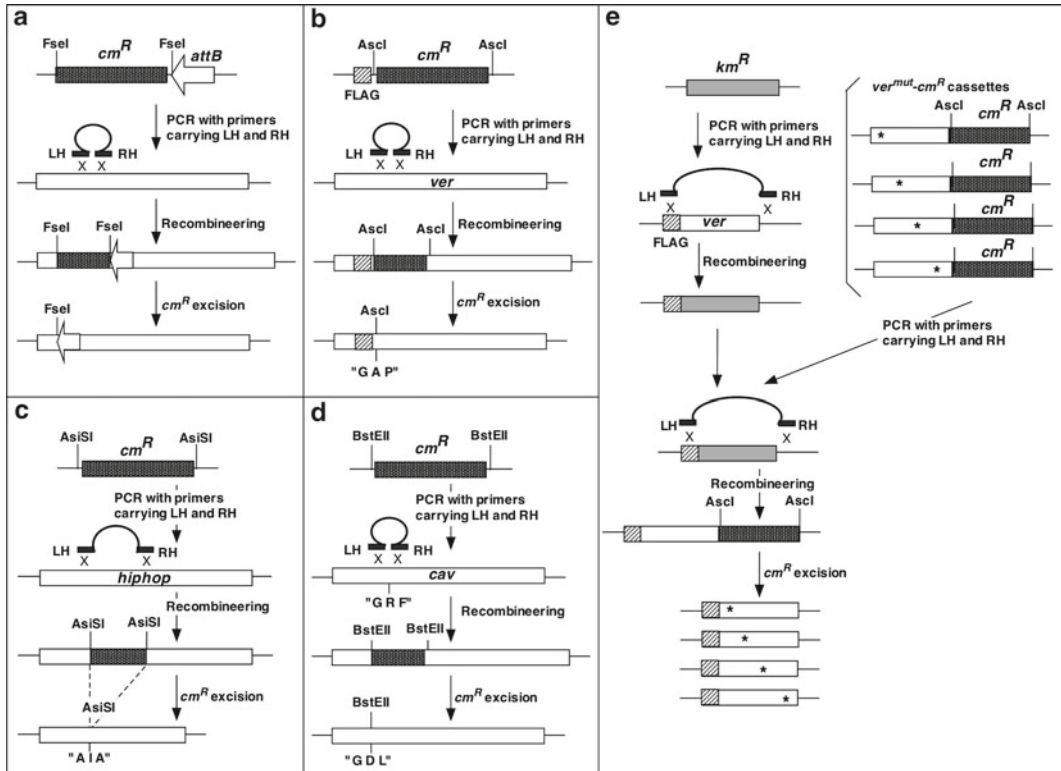
1. MiniPrep.
2. PCR purification.
3. TOPO TA cloning.

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## 3 Methods

### 3.1 A Two-Step Scheme to Modify Multi-copy Plasmids Using Recombineering

For the purpose of vector construction, recombineering enables integration of exogenously provided DNA into a homologous region on the plasmid. This integration is facilitated by the expression of the lambda RED system. In typical recombineering experiments (some



**Fig. 2** Vector construction by recombineering. (a) depicts a scheme for inserting *attB*. At the top is the *cm<sup>R</sup>-attB* cassette as a template for generating the PCR products for recombineering. The PCR product is represented as an  $\Omega$ -shaped object with the two homology arms (LH for left homology and RH for right homology). The two "X" symbols represent points of exchange between the PCR product and the *ver* region (open rectangular box) on the plasmid (not shown). After recombineering, *cm<sup>R</sup>* along with *attB* are inserted into *ver*. The *cm<sup>R</sup>* gene is excised with the *FseI* restriction enzyme. (b) depicts a scheme for introducing an epitope tag. At the top is the FLAG-*cm<sup>R</sup>* template for PCR. At the last step, *cm<sup>R</sup>* is later excised with *Ascl*, which cut site encodes the peptide of "G A P" that serves as a spacer between FLAG (stripped box) and Ver. (c) depicts a scheme for creating an internal deletion mutation. At the top is the *cm<sup>R</sup>* template for PCR. The LH and RH regions are separated by the *hiphop*-coding region to be deleted. After recombineering, the deleted *hiphop* region is replaced with the *cm<sup>R</sup>* gene, which is excised by *AsiSI*. The *AsiSI* site encodes the peptide of "A I A". (d) depicts a scheme for mutating specific residues. At the top is the *cm<sup>R</sup>* template for PCR. The LH and RH regions flank the region that encodes "G R F" in *cav*. After excision of *cm<sup>R</sup>* with *BstEII*, "G R F" is mutated to "G D L". (e) depicts a scheme for introducing multiple mutations into a single locus. In the first step (top left), the entire *ver* coding region is replaced with a *km<sup>R</sup>* gene. In the second step (top right), a *cm<sup>R</sup>* gene is placed next to every *ver* mutations (four shown). These *cm<sup>R</sup>-ver<sup>mut</sup> cm<sup>R</sup>* cassettes serve as PCR templates for the final recombineering step (bottom), in which *km<sup>R</sup>* is replaced with *cm<sup>R</sup>-ver<sup>mut</sup>* followed by *cm<sup>R</sup>* excision. Each mutant allele is also tagged with FLAG

of which are depicted in Fig. 2), the exogenous DNA is a PCR product containing the desired DNA fragment to be inserted flanked by short (50 bp) homology on both sides of the fragment (LH for left homology and RH for right homology in Fig. 2). The sequences of these flanking homologous pieces are identical to those on the plasmid that flank the future insertional site. When dealing with multi-

copy plasmids, it is highly unlikely that every plasmid molecule will acquire an insertion considering that recombination between a PCR product and a circular plasmid is not very efficient. Thus it is essential to use antibiotic selection to recover the desired clone.

We designed a two-step scheme to accomplish efficient modification of multicopy plasmids. In the first step, the desired DNA fragment is physically linked with a selectable marker [e.g., chloramphenicol resistant gene ( $cm^R$ )]. This can be done by PCR with overlapping primers or PCR followed by DNA ligation. Our preferred method is to use recombineering to insert  $cm^R$  next to the desired DNA fragment already cloned into a common cloning vector, such as pCR2.1 from the TOPO TA cloning kit. Unique restriction cut sites are included in the PCR primers to flank the  $cm^R$  marker for its excision in the final cloning step. In the second step, a PCR reaction amplifies the desired DNA fragment along with  $cm^R$ . It integrates, via recombineering, at the desired position on the final plasmid vector, which is then subjected to restriction digestion to excise the  $cm^R$  marker. This is followed by intramolecular ligation giving rise to the final product.

### 3.2 A General Recombineering Protocol

Here we give a detailed protocol for recombineering, which is based on protocols at the Web site:

<http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx>. The readers are encouraged to derive their own modifications of the standard protocol described on the Web site.

#### *Step 1. Prepare PCR insert for recombineering*

Amplify the  $cm^R$  cassette with the desired DNA element using a DNA polymerase with proofreading activities. Use agarose gel electrophoresis to check the specificity and efficiency of the PCR. Purify the PCR product using a commercially available PCR purification kit. Digest the PCR product with DpnI enzyme to destroy template DNA from the plasmid. DpnI only digests methylated DNA so that PCR products are protected. Purify the PCR product after digestion and elute in distilled water.

#### *Step 2. Prepare bacterial cells competent for recombineering*

- 2.1. Inoculate SW102 cells in 5 ml LB growth media with 12.5 µg/ml of tetracycline (tet) at 30 °C overnight (*see Note 1*).
- 2.2. Transfer 500 µl of overnight culture to 25 ml fresh, pre-warmed LB + tet media in a flask larger than 100 ml, and inoculate at 30 °C until OD<sub>600</sub> reaches 0.4–0.6 (about 3 h) (*see Note 2*).
- 2.3. Heat shock the SW102 culture in a 42 °C shaking water bath for exactly 15 min. This is to induce expression of lambda RED.
- 2.4. Chill the culture by placing the flask in ice slurry with frequent mixing. Once the culture is sufficiently chilled (after a few minutes in ice slurry), transfer 10 ml of the cells to

prechilled 15 ml culture tubes. Pellet the cells by centrifugation at  $2,000\times g$  for 5 min at 0 °C (*see Note 3*).

- 2.5. Decant the supernatant. Resuspend the cell pellet in 1 ml of ice-cold, sterile water. Swirl the tube in icy water to achieve fast chilling of the cells. Fill the tube with water and mix by inverting the tube several times. Pellet cells by centrifugation. Repeat this wash three more times.
- 2.6. Carefully pour off the supernatant as the pellet at this stage is very loose, and invert the tubes on a paper towel for a few seconds before returning them to ice. Resuspend the cells in the residual liquid, and keep the tubes on ice until transformation by electroporation.

### *Step 3. Bacterial transformation*

Add ~50 ng of the target plasmid and ~100 ng of the purified PCR product from **step 1** to a cuvette prechilled on ice. The total volume should not exceed 10 % of the cell volume. Add 25–50  $\mu$ l of the competent SW102 cells from **step 2** into the same cuvette, and perform bacterial electroporation according to manufacturer's instructions. Add 500  $\mu$ l of SOC medium, incubate for 1 h at 30 °C, and plate the entire culture on LB + cm (17  $\mu$ g/ml) plates. Incubate overnight at 30 °C.

### *Step 4. Post-transformation cleanup and validation*

- 4.1. Isolate DNA from small cultures inoculated from several cm<sup>R</sup> colonies grown overnight at 30 °C. Perform restriction digests to confirm the overall structure of the recovered plasmids by comparing it to a similar digestion of the starting plasmid. Most colonies would yield a DNA mixture of both the desired plasmid with a cm<sup>R</sup> insertion and the original unmodified plasmid (*see Note 4*).
- 4.2. To clean up the mixture, dilute the miniprep DNA 1:200–1,000 in water. Transform 1  $\mu$ l of the diluted DNA into cells of a standard bacterial cloning strain and plate on LB + cm plates. Purify DNA from several colonies per plate, and perform restriction digest to identify colonies that no longer contain the original non-cm<sup>R</sup> plasmid.

### *Step 5. cm<sup>R</sup> marker excision*

Digest several nanograms of the “cleaned-up” plasmid with the restriction enzyme, which cut sites have been previously engineered to flank the cm<sup>R</sup> marker. Perform ligation after heat inactivation of the restriction enzyme. Transform the ligation reactions into cells of a standard bacterial cloning strain, and plate the cells on plates with the appropriate antibiotics for the target plasmid [e.g., ampicillin (amp)]. Perform restriction digest validation of several clones and DNA sequencing to validate intact DNA elements if necessary.

### 3.3 An Alternative Recombineering Protocol

The protocol described in Subheading 3.2 has been referred to as the “co-transformation” protocol in which the target plasmid and the donor PCR products are transformed simultaneously into bacterial cells made competent for recombineering. However, due to the large amount of DNA required and the relatively small volume of total DNA allowed, this might not be practical for all situations. Alternatively, one can introduce the target plasmid and the donor PCR products in two transformation steps.

#### *Step 1. Preparing competent SW102 cells*

When used for transformation of the target plasmid, SW102 cells should not be heat shocked to induce lambda RED. Cells can be made competent according to **steps 2.1–2.6** in Subheading 3.2 with **step 2.3** omitted. In **step 4** instead of using only 10 ml of the cells, the entire culture can be used for preparing competent cells as unused cells will be stored for future uses (*see Note 5*).

#### *Step 2. Target plasmid transformation*

Transform a few nanograms of the target plasmid into competent SW102 cells by electroporation as described in **step 3** of Subheading 3.2, and plate the cells on plate with the appropriate antibiotics for the target plasmid (e.g., amp).

#### *Step 3. Recombineering with PCR products*

Perform recombineering as described in **steps 2** and **3** in Subheading 3.2 with the following changes:

1. For **steps 2.1** and **2.2**, when culturing SW102 cells with the target plasmid, use the appropriate antibiotics for the target plasmid.
2. For **step 3**, omit DNA from the target plasmid and reduce the amount of the PCR product to about 50 ng.

### 3.4 Generating the Master Construct with an attB Attachment Site

In performing SIRT-mediated gene manipulation in *Drosophila*, all genetic modifications of the target gene are to be introduced as plasmids carrying the *attB* attachment site. We routinely construct a master clone with *attB* inserted at the desired position in the plasmid (i.e., identical to the position where *attP* has been introduced onto the chromosome, Fig. 1). All subsequent modifications are made to this master clone.

The construction of this *attB*-containing master clone is accomplished with the two-step scheme described in Subheading 3.1. Below we described this cloning step in detail using an example in which we constructed a master clone for modifying the *Drosophila ver* gene. Figure 2a is a schematic representation of this experiment.

For PCR amplification of *attB*, we use an existing *cm<sup>R</sup>* cassette in which an *attB* site was cloned adjacent to a *cm<sup>R</sup>* gene that is flanked by FseI restriction cut sites [7]. The primers used are:



## 1. ver4401Cm:

aataagtaaaaattagcaggggtagtcaaaacaactgaaaatttgtaaGGCCG-GCCctgtggaacaccc

The 50 bp sequence in lower case is homologous to the left side of the position where we plan to insert *attB* (LH in Fig. 2a). The *FseI* site is in upper case. Sequence in italics is homologous to *cm<sup>R</sup>* (see Note 6).

## 2. ver4500attB:

cagggtcacattaatttgcagaaccgcgcaatattttcttttaacccccCGACAT-GCCCGCCGTGACCG

The 50 bp sequence in lower case is homologous to the right side of the *attB* insertion site at *ver* (RH in Fig. 2a). The 20 bp sequence in upper case is homologous to *attB*.

After PCR amplification of the *cm<sup>R</sup>-attB* cassette with the above primers, the PCR products are transformed into SW102 cells that carry the target plasmid pTV[*ver*], which contains a genomic fragment of *ver* sub-cloned into a generic gene targeting vector. Using recombineering protocols described in Subheading 3.2 or 3.3, recover clones with *cm<sup>R</sup>-attB* inserted into pTV[*ver*]. After cleaning up (step 4 in Subheading 3.2), *cm<sup>R</sup>* was excised by an *FseI* digestion followed by plasmid re-ligation. This generates the plasmid pTV[*ver-attB*] (see Note 7).

### 3.5 Inserting an Epitope Tag

SIRT can be used to introduce epitope tags to an endogenous locus. Again using the *ver* locus as an example, we describe a protocol to insert a FLAG tag to the N-terminus of Ver. Figure 2b is a schematic representation of this experiment.

#### Step 1. Generating a plasmid containing a Flag-cm cassette

##### 1.1. Use the following pair of primers to amplify a DNA fragment that contains a FLAG-encoding fragment followed by a *cm<sup>R</sup>* gene that is flanked two *AscI* cut sites.

## 1. Flag-AscI-Cm:

gactacaaagacgatgacgacaagGGCGCGCCagccagtatacactc-cgcta Sequence in lower case encodes FLAG. The *AscI* site is in upper case. The italicized sequence in lower case is homologous to *cm<sup>R</sup>*.

## 2. AscI-Cm:

GGCGCGCC ctgtggaacacctacatctg

The *AscI* site is in upper case. The italicized sequence in lower case is homologous to *cm<sup>R</sup>*.

##### 1.2. Clone the above PCR product using the TOPO cloning kit from Invitrogen according to manufacturer's instruction. Use LB + cm plates to select for the correct clones, which are subjected to sequencing to confirm the integrity of the FLAG tag and *AscI* cut sites.



*Step 2: Inserting a FLAG tag N-terminal to Ver*

- 2.1. Use the following primers to amplify a *Flag-cm<sup>R</sup>* fragment flanked by small regions of homology. The template was the *Flag-cm<sup>R</sup>* cassette constructed at **step 1** of Subheading 3.5.

1. ver4640-Flag-L:

gcactgcaataaagaaatcccctttgaaatcgagactaagcaatagaatg-  
GACTACAAAGACGATGACGAC

Sequence in lower case is homologous to *ver*(LH in Fig. 2b). Sequence in upper case is homologous to FLAG.

2. ver4691-Cm-R:

acgaagtatccagctggctttctatgtcctcgaaactctgataaaatc**TGGC**-  
GCG CCctgtggaacac.

Sequence in lower case is homologous to *ver* (RH in Fig. 2b). In this primer, the *AscI* cut site (GGCGCGCC) is preceded with a “T” in bold. Since the *AscI* cut site is 8 bp in length, an extra “T” has been added to ensure that the FLAG tag is in frame with the rest of the Ver protein. The sequence **TGGCGCGCC** when translated in the reverse direction encodes a peptide of Gly Ala Pro (“G A P” in Fig. 2b), which also serves as a spacer between FLAG and Ver (*see Note 8*).

- 2.2. Using the recombineering protocols described in Subheading 3.2 or 3.3, insert this *Flag-cm<sup>R</sup>* fragment into the master clone of pTV[ver-attB] generated from Subheading 3.4. Use *AscI* to excise the *cm<sup>R</sup>* gene. This gives rise to the plasmid pTV[FLAG-ver-attB].

### 3.6 Creating In-Frame Deletion Mutations

An efficient way to identify critical domains for protein function is to create in-frame deletion mutations that encode truncated proteins missing different domains. Using the *hiphop* locus as an example, we describe a protocol to create an internal deletion eliminating about one third of the protein (Fig. 2c). The scheme involves replacing the DNA fragment to-be-deleted with a *cm<sup>R</sup>* gene flanked by restriction sites using recombineering. The *cm<sup>R</sup>* gene is then excised resulting in replacing the deleted region with the restriction site (*see Note 9*).

*Step 1.* Use the following primers to amplify a *cm<sup>R</sup>* gene flanked by 50 bp fragments homologous to the *hiphop* locus. The 50 bp homologous pieces (LH and RH in Fig. 2c) are taken from positions in the HipHop-coding region immediately adjacent to either side of the future deletion as shown in Fig. 2c.

1. HipHopA2As/SICm-Forward

gccagggagactgccgcgagcattacggacgtcagcggcagtcagtcacgcGC-  
GATCGCaggaggacagctgatagaa

Sequence in lower case is homologous to *hiphop* (LH in Fig. 2c). The *AsiSI* cut site is in upper case. The lower case and italicized sequence is homologous to *cm<sup>R</sup>*.

## 2. HipHopA2AsiSICm-Forward

gtgccattcacggcggttcaaactgagattcgagttgggggtcgtagtcgtc**AGC**-  
GATCGCctgtggaacacctacatct

Sequence in lower case is homologous to *hiphop* (RH in Fig. 2c). The *AsiSI* cut site is in upper case. The “A” in bold preceding the *AsiSI* cut site is included to preserve reading frame. The sequence AGCGATCGC when translated in the reverse orientation encodes the peptide Ala Ile Ala (“A I A” in Fig. 2c). The lower case and italicized sequence is homologous to *cm<sup>R</sup>*.

*Step 2.* Using the recombineering protocols described in Subheading 3.2 or 3.3, insert this *cm<sup>R</sup>* fragment into the master clone of pTV[hiphop-attB]. Use *AsiSI* to excise *cm<sup>R</sup>*.

## 3.7 Site Directed Mutagenesis with Recombineering

Besides making large deletion of the coding region, mutating conserved residues by site-directed mutagenesis is another common way to dissect protein function. Using the *cav* locus as an example, we describe a protocol to mutate individual residues (Fig. 2d). The scheme involves replacing individual residue(s) of interest with a *cm<sup>R</sup>* gene flanked by restriction sites. The *cm<sup>R</sup>* gene is excised following recombineering essentially replacing the target residue(s) with the restriction site (*see Note 9*).

*Step 1.* Use the following primers to amplify a *cm<sup>R</sup>* gene flanked by 50 bp fragments homologous to the *cav* locus.

## 1. HOAP89R:DBstEICm-Forward

atgaccgcttggaattgtctgtgggaagccaaaagaggttgaagcaaaaGGT-  
GACCaggaggacagctgatagaa

Sequence in lower case is homologous to *cav*(LH in Fig. 2d). The *BstEII* cut site is in upper case. The lower case and italicized sequence is homologous to *cm<sup>R</sup>*. The *BstEII* enzyme is a 7 bp cutter with the sequence GGTNACC where N represents any base. Due to this ambiguous nucleotide, various amino acid combinations can be created.

## 2. HOAP89R:DBstEIIcM-Reverse

gcgcaccgctttcatatacatctcggttgatgaatctctcagacttggttcac  
AAGGTCACCctgtggaacacctacatct

Sequence in lower case is homologous to *cav*(RH in Fig. 2d). The *BstEII* cut site is in upper case. The “AA” in bold is included to preserve reading frame. The lower case and italicized sequence is homologous to *cm<sup>R</sup>*.

We wish to change the amino acid Gly Arg Phe (“G R F” in Fig. 2d) of the Cav protein to Gly Asp Leu (“G D L” in Fig. 2d) where Gly and Arg are conserved residues. We choose to add two adenosines immediately upstream of the *BstEII* cut site in the reverse primer. This puts the coding sequence in frame and creates

our desired mutation. The sequence AAGGTCACC when translated in the reverse orientation encodes Gly Asp Leu.

*Step 2.* Using the recombineering protocols described in Subheading 3.2 or 3.3, insert this  $cm^R$  PCR product into the master clone. Use BstEII enzyme to excise the  $cm^R$  gene.

### 3.8 One-Step Introduction of Multiple Mutations

The site-directed mutagenesis protocol described in Subheading 3.7 is limited by the number of suitable restriction enzymes. This limitation is placed not only on the residues that can be mutated, also on the exact amino acid to which a particular residue can be mutated. Situations exist in which a series of random mutations have been selected based on functional assays *ex vivo* and that the introduction of these mutations into the intact organism would be rather illuminating on gene function. Using the *ver* locus as an example, we describe a protocol for introducing a series of point mutations that we recovered from a yeast two-hybrid assay into the master clone of pTV[Flag-Ver-attB] generated in Subheading 3.5, and doing so with a single set of primers.

Figure 2c is a schematic representation of this experiment. The scheme involves first replacing the entire *ver* coding region in the pTV[Flag-ver-attB] master clone with a kanamycin-resistant ( $km^R$ ) gene. Secondly, a series of cassettes are constructed in which a  $cm^R$  gene, excisable by restriction digest, is placed next to the *ver* coding region for each *ver* mutation ( $ver^{mut}$ ) cloned into the pBTM vector. Thirdly, the  $ver^{mut}$ - $cm^R$  cassettes are introduced as PCR products by recombineering, replacing the  $km^R$  gene in the master clone. After  $cm^R$  excision, a series of plasmids are generated each containing a different mutation.

It is necessary to replace the coding region of *ver* in the master clone of pTV[Flag-ver-attB] in the first step. Otherwise the  $ver^{mut}$ - $cm^R$  PCR products would share extensive homology (the entire *ver* coding region) with pTV[Flag-ver-attB], which would make it difficult to predict the exact point of exchange between the plasmid and the PCR product ("X" in Fig. 2c). This would necessitate a cumbersome screening step by DNA sequencing to identify clones with the desired mutations. By limiting the exchange points to a 50 bp region to either side of the *ver* coding region, our scheme ensures the recovery of mutations in all clones after recombineering.

*Step 1. Replacing ver with  $km^R$  in the plasmid pTV[Flag-ver-attB]*

1.1. Use the following primers to amplify a  $km^R$  gene using the pCR2.1 vector as the PCR template.

1. ver4654L1-Flag-Km:

gaaatcgcgactaagcaaataagaatggactacaagacgatgacgacaagT-  
GCTAAAGGAAGCGGAACAC

Sequence in lower case is homologous to the 5' region of *ver* including the FLAG tag in pTV[Flag-ver-attB] (LH in Fig. 2c). Sequence in upper case is homologous to  $km^R$ .

## 2. ver5320-R1-Km:

tttgaatttttattaccagtaaaatttcaatacaaaaaaccaacgatactaG-  
GTGAGCAAAAACAGGAAGG

Sequence in lower case is homologous to the 3' region of *ver* (RH in Fig. 2e). Sequence in upper case is homologous to *km<sup>R</sup>*.

- 1.2. Using the recombineering protocols described in Subheading 3.2 or 3.3, replace the *ver* coding region in pTV[Flag-ver-attB] with this *km<sup>R</sup>* fragment. Use *km* as the selectable marker for recombineering (see Note 10).

*Step 2. Generating a *cm<sup>R</sup>* cassette for each *ver* mutation*

- 2.1. Use the following primers to amplify a *cm<sup>R</sup>*.

## 1. ver-end-L2-Cm-F:

gaccagctttctgtacaaagtgggtgatggggatccgtcgacctg-  
cagGGCGCGCC*Cagccagtatacac*

## 2. ver-end-R2-Cm-R:

ttaataataaaaatcataaatcataagaattcgcccggaattagcttgg  
GGCGCGCC*ctgtggaacacc*

In these primers, sequence in lower case is homologous to vector sequences right after the stop codon of *ver*. The AscI sites are in upper case. The italicized sequence in lower case is homologous to *cm<sup>R</sup>* (see Note 11).

- 2.2. Using the recombineering protocols described in Subheading 3.2 or 3.3, insert this *cm<sup>R</sup>* fragment into pBTM clones with the *ver* mutations.

*Step 3: Generating the final plasmid of pTV[FLAG-*ver<sup>mut</sup>*-attB]*

- 3.1. Use the following primers to amplify the *ver<sup>mut</sup>-cm<sup>R</sup>* cassette for each *ver<sup>mut</sup>*, using template plasmids generated in step 2 of Subheading 3.8.

## 1. 4654L1-Flag-ver-F:

gaaatcgagactaagcaaatagaatggactacaaagacgatgacga-  
caagGATTTTAATCAGAGTTTCGAG

Sequence in lower case is homologous to the 5' region of *ver* including the FLAG tag in pTV[FLAG-Ver-attB] (LH in Fig. 2e). Sequence in upper case is homologous to the start of *ver* coding region downstream of the start codon.

## 2. ver5320-R1-cm:

tttgaatttttattaccagtaaaatttcaatacaaaaaaccaacgatactaG-  
GCGCGCC*ctgtggaacacc*

Sequence in lower case is homologous to the 3' region of *ver* (RH in Fig. 2e). The AscI cut site is in upper case. Italicized sequence in lower case is homologous to *cm<sup>R</sup>*.

- 3.2. Using the recombineering protocols described in Subheading 3.2 or 3.3, replace the *km<sup>R</sup>* gene in the construct generated in **step 1** with these *ver<sup>mut</sup>-cm<sup>R</sup>* cassettes. Use *AscI* to excise *cm<sup>R</sup>*. Sequence the clones to ensure the integrity of the *ver* coding region and the presence of the desired point mutations.

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## 4 Notes

1. It is important to grow SW102 cells under 32 °C. Higher temperature will result in premature activation of the lambda RED system.
2. We have performed successful recombineering experiments with an OD value as low as 0.3 or as high as 0.7.
3. From this step on, keep the cells on ice at all time and use prechilled solutions.
4. We usually use the *EcoRI* enzyme since the *cm<sup>R</sup>* marker introduces an additional *EcoRI* site.
5. SW102 cells made this way can be stored at –80 °C for future uses. Substitute water with 10 % sterile glycerol at the final washing step, and aliquot unused cells into tubes for storage at –80 °C.
6. Both *FseI* site and the sequence in italics will anneal to the *cm<sup>R</sup>-attB* cassette.
7. *FseI* is not stable at –20 °C, and should be stored at –80 °C.
8. Because the *Flag-cm<sup>R</sup>* cassette does not carry an ATG codon, this fragment has to be inserted downstream of the endogenous ATG codon.
9. Care needs to be taken to preserve the correct reading frame when using a restriction enzyme that does not have a 6 bp cut site.
10. This recombineering reaction involves the replacement of a DNA fragment with another. It is very important to sequence several clones to ensure the integrity of the recombineering junctions.
11. This *cm<sup>R</sup>*-homologous sequence is shorter than one would normally use for PCR amplification since we use a *cm<sup>R</sup>* gene already flanked by *AscI* as the PCR template so that the *AscI* site in the oligos also serves as a part of the primer.

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Gene Correction

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