Chapter 26

Recombination-Mediated Genetic Engineering of Large Genomic DNA Transgenes

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Abstract

Faithful gene activity reporters are a useful tool for evo-devo studies enabling selective introduction of specific loci between species and assaying the activity of large gene regulatory sequences. The use of large genomic constructs such as BACs and fosmids provides an efficient platform for exploration of gene function under endogenous regulatory control. Despite their large size they can be easily engineered using in vivo homologous recombination in *Escherichia coli* (recombineering). We have previously demonstrated that the efficiency and fidelity of recombineering are sufficient to allow high-throughput transgene engineering in liquid culture, and have successfully applied this approach in several model systems. Here, we present a detailed protocol for recombineering of BAC/fosmid transgenes for expression of fluorescent or affinity tagged proteins in *Drosophila* under endogenous in vivo regulatory control. The tag coding sequence is seamlessly recombineered into the genomic region contained in the BAC/fosmid clone, which is then integrated into the fly genome using φC31 recombination. This protocol can be easily adapted to other recombineering projects.

Key words: Red/ET, Recombineering, FlyFos

1. Introduction

Recombineering (recombination-mediated genetic engineering, also known as Red/ET cloning) is a technique for DNA engineering in *Escherichia coli*. Recombineering relies on the highly efficient homologous recombination activities mediated by the products of the RecE and RecT genes from *E. coli sbcA* strain (1) or Red alpha and Red beta genes originating from phage λ (2). In typical recombineering experiment, a linear fragment of DNA,

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usually containing an antibiotic resistance marker, flanked by short (30–50 bp) regions of homology is transformed into recombineering proficient *E. coli* cells containing the target sequence, which can be the bacterial genome itself or an episomal construct such as plasmid, fosmid, BAC, or PAC. The homologous recombination reaction then occurs within minutes, and the recombination product is selected for by overnight growth on selective media.

Since recombineering is not constrained by specific sequence requirements such as the presence of restriction sites, it provides a great freedom and flexibility and has been applied to broad range of DNA engineering tasks (3–5). Recently, we demonstrated that recombineering can be optimized for multistep engineering in liquid culture, without the need to isolate and verify the intermediate products. We have successfully applied this high-throughput approach for engineering of BAC/fosmid clones into transgenes for expression of fluorescent- and/or affinity-tagged proteins under their endogenous control in *Caenorhabditis elegans* (6), mammalian cells (7), and *Drosophila* (8). As fosmid/BAC library can be easily generated (see Chapter 25), this approach is generally applicable for any model organism of interest. Recently, we have created a fosmid library that can be used for straight-forward generation of transgenic fly lines using φC31 site-specific integration (8, 9). Here, we present the detailed protocol for engineering of *Drosophila* genomic fosmid clones into tagged transgenes (8) by combination of Red/ET recombineering and Flp/FRT site-specific recombination. The recombineering pipeline consists of three steps, where (1) host strain carrying targetted fosmid is transformed with recombineering helper, thus rendering bacteria recombineering-competent; (2) PCR-amplified recombineering cassette is introduced into induced bacteria expressing recombineering genes, and (3) selectable marker included in the recombineering cassette is removed using flipase (Fig. 1). The protocol allows for simultaneous processing of multiple samples in microcentrifuge tubes or in 96-well plate format. This protocol can be easily adapted to other recombineering projects.

2. Materials

2.1. Amplification of the Tagging Cassette

1. Phusion®; high-fidelity DNA polymerase (NEB).
2. 5× Phusion™ HF buffer.
3. 10 mM dNTPs.
4. 96-Well thermal cycler.
5. PureLink™ PCR purification kit (Invitrogen).
6. Nuclease-free water (see Note 1).

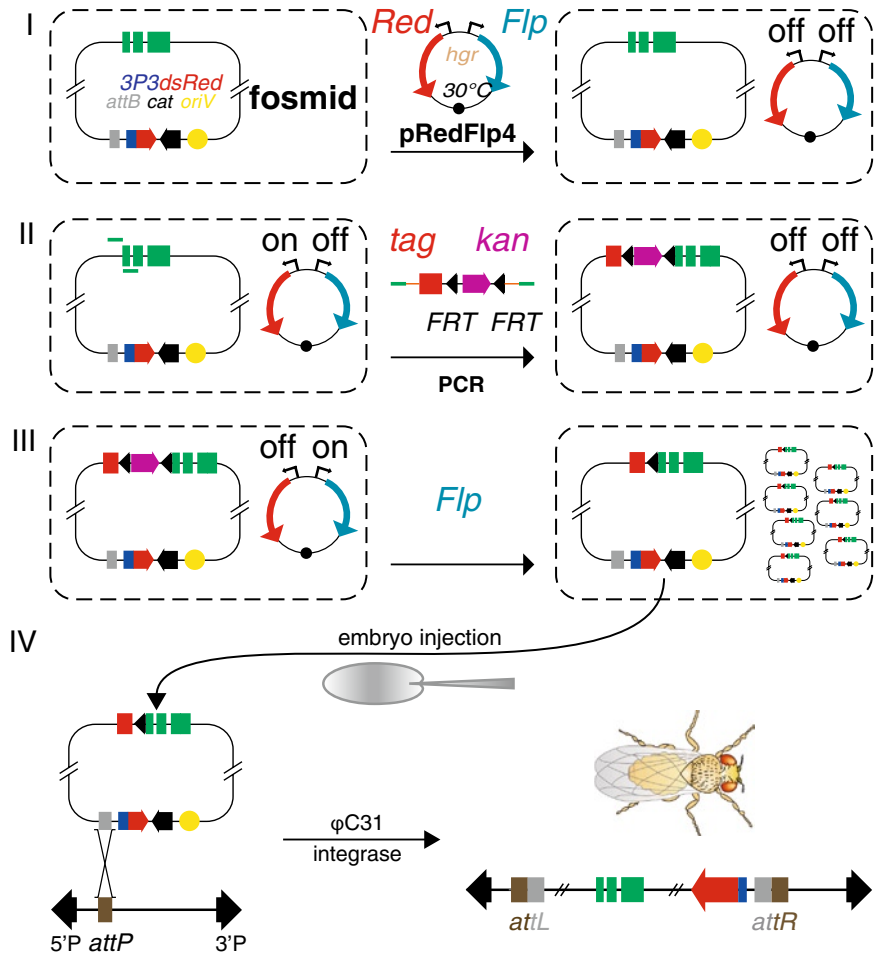

Fig. 1. The recombineering pipeline. *Escherichia coli* cultures containing a fosmid clone of interest are rendered recombineering proficient by transformation of the pRedFlp4 plasmid, which carries both the Red operon genes and the Flp recombinase under independently inducible promoters. (**a**) After L-rhamnose-induced Red operon expression, a PCR product carrying 50 bp homology arms surrounding the tag and the FRT-flanked kanamycin resistance gene (*kan*) is electroporated into the cells. Only recombinant fosmids are able to grow efficiently in the presence of kanamycin (**b**) The *kan* gene is removed by inducing Flp expression with anhydrotetracycline on the pRedFlp4 plasmid, leaving the tagged transgene with a residual FRT sequence on the gene–tag boundary. (**c**) Finally, the clone is induced to high copy and injected into flies carrying an *attP* landing site for targeted integration (**d**).

2.2. Transformation of pRedFlp4 Recombineering Helper

1. 10% Glycerol in Milli-Q grade water (see Note 2).
2. SOC medium (see Note 3).
3. LB + chloramphenicol (25 µg/ml).
4. YENB (0.75% bacto yeast extract, 0.8% nutrient broth) + chloramphenicol (25 µg/ml) (see Note 4).
5. LB + chloramphenicol (25 µg/ml) + hygromycin (50 µg/ml) (see Note 5).

6. 96-Well deep square well plates (2 ml)/microcentrifuge tubes (2 ml).
7. Air-permeable seal.
8. Shaker for deep well plates/thermomixer (Eppendorf).
9. Centrifuge for deep well plates/microcentrifuge (Eppendorf).
10. 96-Well electroporator (Harvard Apparatus)/electroporator (BioRad).
11. 96-Well electroporation cuvette (Harvard Apparatus)/electroporation cuvette (BioRad).

2.3. Tagging by Red/ET Recombination

1. 10% Glycerol in Milli-Q grade water.
2. SOC medium.
3. YENB + chloramphenicol (25 μg/ml) + hygromycin (50 μg/ml).
4. LB + chloramphenicol (25 μg/ml) + hygromycin (50 μg/ml) + kanamycin (25 μg/ml).
5. 25% L-rhamnose in water.
6. 96-Well deep square well plates (2 ml)/microcentrifuge tubes (2 ml).
7. Air-permeable seal.
8. Shaker for deep well plates/thermomixer (Eppendorf).
9. Centrifuge for deep well plates/microcentrifuge (Eppendorf).
10. 96-Well electroporator (Harvard Apparatus)/electroporator (BioRad).
11. 96-Well electroporation cuvette (Harvard Apparatus)/electroporation cuvette (BioRad).

2.4. Removal of the Selectable Marker and pRedFlp Helper

1. LB + chloramphenicol (25 μg/ml).
2. LB + chloramphenicol (25 μg/ml) + hygromycin (50 μg/ml) + anhydrotetracycline (200 nM).
3. 96-Well deep square well plates (2 ml)/microcentrifuge tubes (2 ml).
4. Air-permeable seal.
5. Shaker for deep well plates/thermomixer (Eppendorf).

2.5. Verification of the Tagging Result

1. High-throughput miniprep kit (see Chapter 25).
2. DNA sequencing.

2.6. DNA Purification and Fly Transformation

1. LB + chloramphenicol (25 μg/ml).
2. LB + chloramphenicol (25 μg/ml) + l-arabinose (0.1%).
3. Plasmid Maxi Kit (QIAGEN).
4. Whatmann filter paper.

5. 100% Isopropanol.
6. 70% Ethanol.
7. 1× TE, pH 8.0.
8. Nuclease-free water.

3. Methods

Liquid culture recombineering can be preformed in both Eppendorf tubes and 96-well plates using the same protocol. Format-specific differences are indicated in the text below.

3.1. Amplification of the Tagging Cassette

In this step, the linear tagging cassette is amplified from the tagging vector (Fig. 2). The cassette is flanked by homology arms targeting it to the desired location.

1. Design recombineering primers for each sample. Primers include a priming part (20–25 bp) that is complementary to the ends of the tag sequence (forward and reverse) on 5′ end and 50 bp homology arms complementary to the target sequence (see Note 6). Verify the orientation of both primers.
2. Set up 50 µl PCRs to amplify the tagging cassettes (see Note 7). Use 25–50 ng of the tagging vector as a template. Use HPLC-purified recombineering primers at 10 nmol/µl final concentration. Run the PCR for 20–25 cycles (see Notes 1 and 8).
3. Verify the PCR by running 5 µl of the reaction on an agarose gel.
4. Purify the DNA with (96-well) PCR purification kit following the manufacturer's instructions (see Note 9). Elute DNA with 500 µl of nuclease-free water.
5. Store the amplified tagging cassettes at − 20 °C.

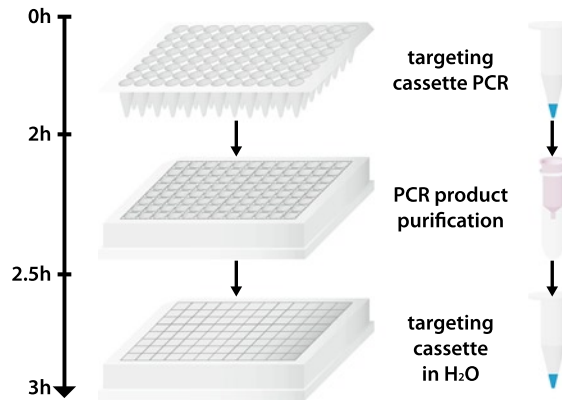

Fig. 2. Amplification of the tagging cassette.

3.2. Transformation of pRedFlp4 Recombineering Helper

In the following steps, the recombineering helper that encodes Red operon and flipase is introduced into the bacteria by electroporation (Figs. 1a and 3).

1. Use glycerol stocks to inoculate 1 ml of LB + Cm in a 96-well deep well plate/2 ml tube (see Note 10). Seal the plate with an air-permeable seal and culture overnight at 37°C with vigorous shaking. If using single tube, puncture the lids with a sterile needle.

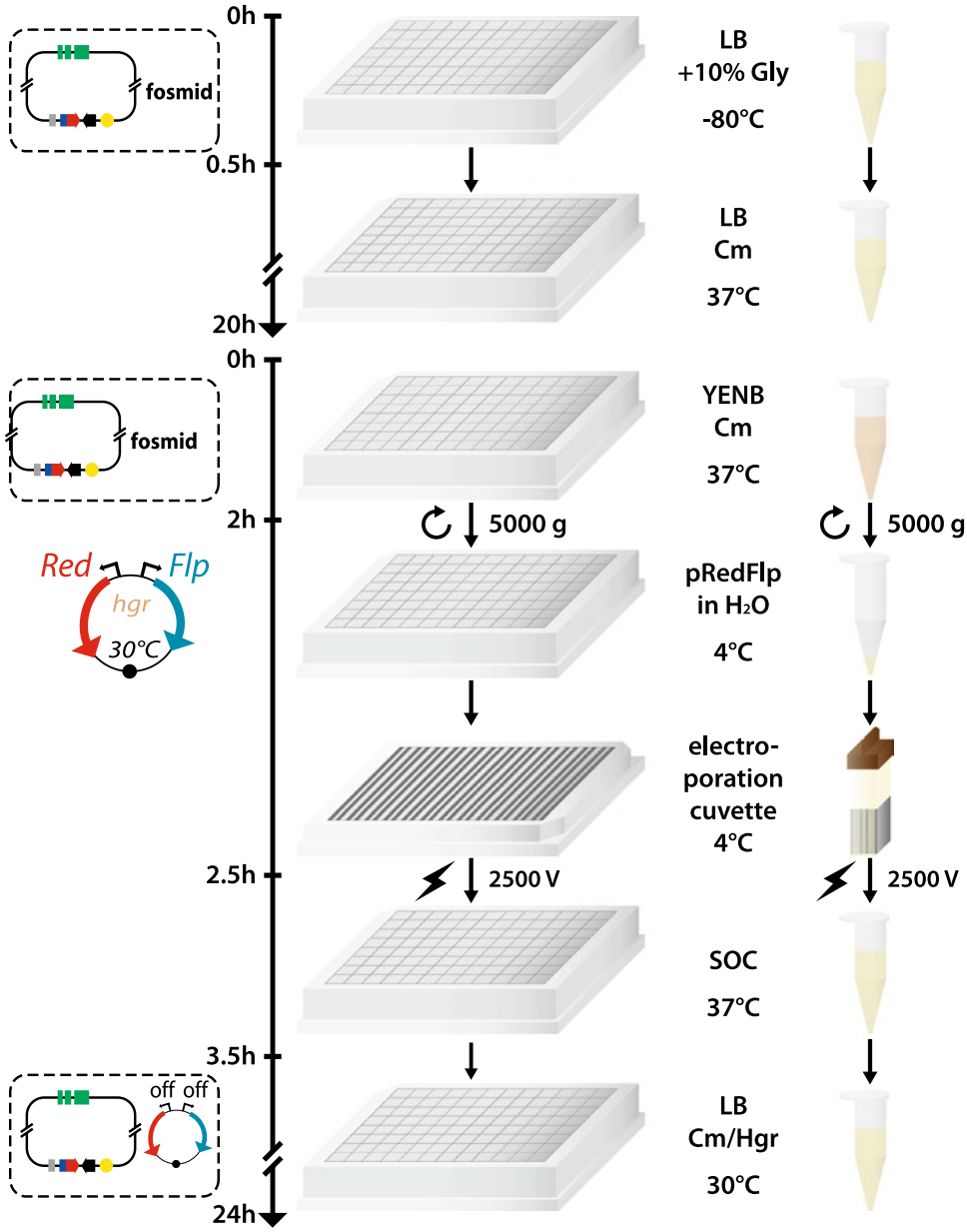

Fig. 3. Transformation of the recombineering helper.

2. Use 40 µl of the overnight cultures to inoculate 1 ml of YENB + Cm per well/tube. Seal the plate with an air-permeable seal and culture cells for 2 h at 37 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.
3. Centrifuge the plate/tube at 5,000 × *g* for 10 min at 2 °C. Discard the supernatant and blot any media residues on a stack of paper towels.
4. Add 1 ml of ice-cold 10% glycerol into each well/tube. Seal the plate with an aluminum or plastic seal.
5. Resuspend the bacteria by shaking the plate at 1,400 rpm for 1 min at 2 °C or by vortexing the tube.
6. Centrifuge the plate/tube at 5,000× *g* for 10 min at 2 °C. Discard the supernatant and blot any media residues on a stack of paper towels.
7. Add 100 µl of pRedFlp4 (0.1 ng/µl in ice-cold water) into each well/tube. Resuspend the bacteria by pipetting.
8. Transfer the cell suspension into a chilled (96-well) electroporation cuvette and electroporate at 2,500 V (see Note 11).
9. Immediately transfer the cell suspension into a new plate/tube with 1 ml of SOC per well/tube.
10. Seal the plate with an air-permeable seal and culture for 1 h at 37 °C (see Note 12) with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.
11. Use 100 µl of the transformed bacteria to inoculate 1 ml of LB + Cm + Hgr per well/tube. Seal the plate with an air-permeable seal and culture overnight at 30 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.

3.3. Tagging by Red/ET Recombination

In the following steps, the Red-mediated homologous recombination is performed (Figs. 1b and 4). l-Rhamnose induces Red operon expression and thus homologous recombination. Then the PCR product is electroporated into the cells. This PCR product contains a kanamycin resistance gene. In the last step, recombinant fosmids are selected on kanamycin. Two controls can help in the evaluation of the success of each step prior to clone validation: a "no tagging cassette" control and a "no Red induction" control.

1. Use 40 µl of the overnight cultures to inoculate 1 ml of YENB + Cm + Hgr per well/tube.
2. Seal the plate with an air-permeable seal and culture cells for 2 h at 30 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.
3. Induce Red operon expression by adding 20 µl of 25% l-rhamnose into each well/tube.

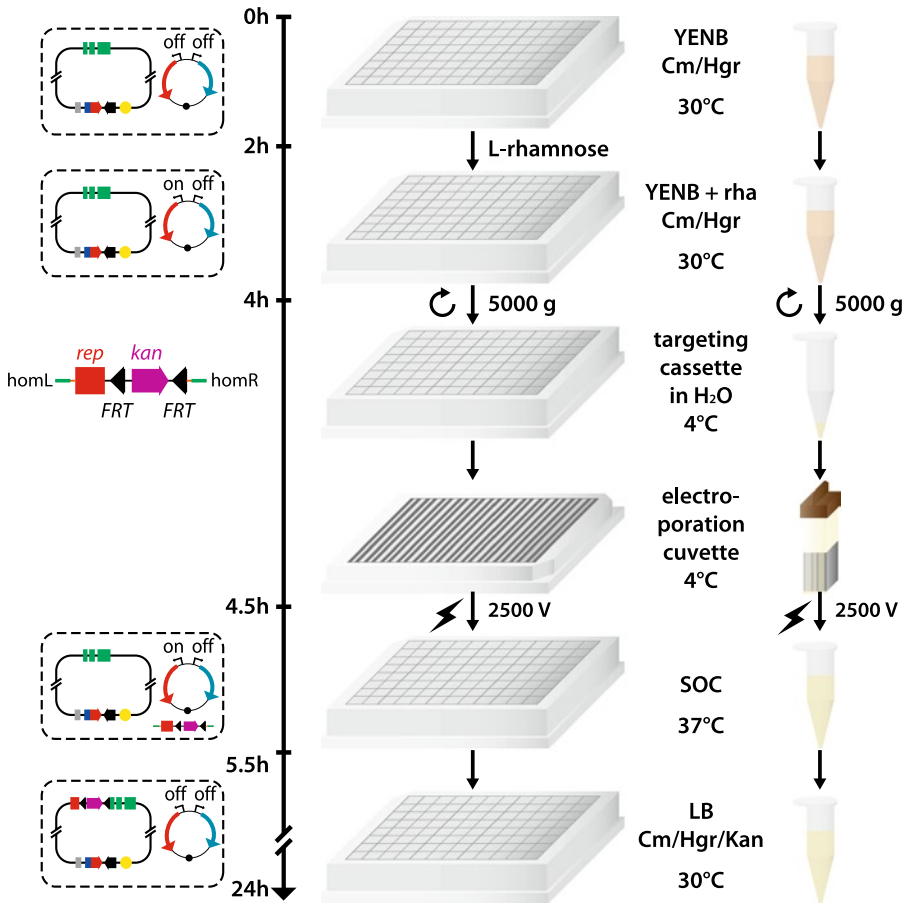

Fig. 4. Tagging by homologous recombination.

4. Seal the plate with an air-permeable seal and incubate plate/tube for 2 h at 30°C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.
5. Centrifuge the plate/tube at 5,000× *g* for 10 min at 2°C. Discard supernatant and blot any media residues on a stack of paper towels.
6. Add 1 ml of ice-cold 10% glycerol into each well/tube. Seal the plate with an aluminum or plastic seal.
7. Resuspend the bacteria by vigorously shaking the plate for 1 min at 2°C or by vortexing the tube.
8. Centrifuge the plate/tube at 5,000× *g* for 10 min at 2°C. Discard the supernatant and blot any media residues on a stack of paper towels.
9. Add 100 µl of the tagging cassette (5 ng/µl in ice-cold water) into each well/tube. Resuspend cells by pipetting.

10. Transfer the cell suspension into a chilled (96-well) electroporation cuvette and electroporate at 2,500 V.
11. Immediately transfer the cell suspension into a new plate/tube with 1 ml of SOC per well/tube.
12. Seal the plate with an air-permeable seal and culture for 1 h at 37 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.
13. Use 100 µl of the transformed bacteria to inoculate 1 ml of LB + Cm + Hgr + Kan per well/tube. Seal the plate with an air-permeable seal and culture overnight at 30 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle (see Notes 13 and 14 for suitable efficiency controls at this step).

3.4. Removal of the Selectable Marker and pRedFlp Helper

In the following steps, the FRT-flanked selectable marker is removed (Figs. 1c and 5). Anhydrotetracyclin (AHT) induces Flippase (Flp) expression. This enzyme catalyzes recombination between Flippase Recognition Target (FRT) sites and thus removes the kan gene. The pRedFlp plasmid (6) has a temperature-sensitive origin of replication from pSC101 (1). In the last step, overnight growth at 37 °C instead of 30 °C removes pRedFlp plasmids from the cells.

1. Use 10 µl of the overnight cultures to inoculate 1 ml of LB + Cm + Hgr + AHT per well/tube.

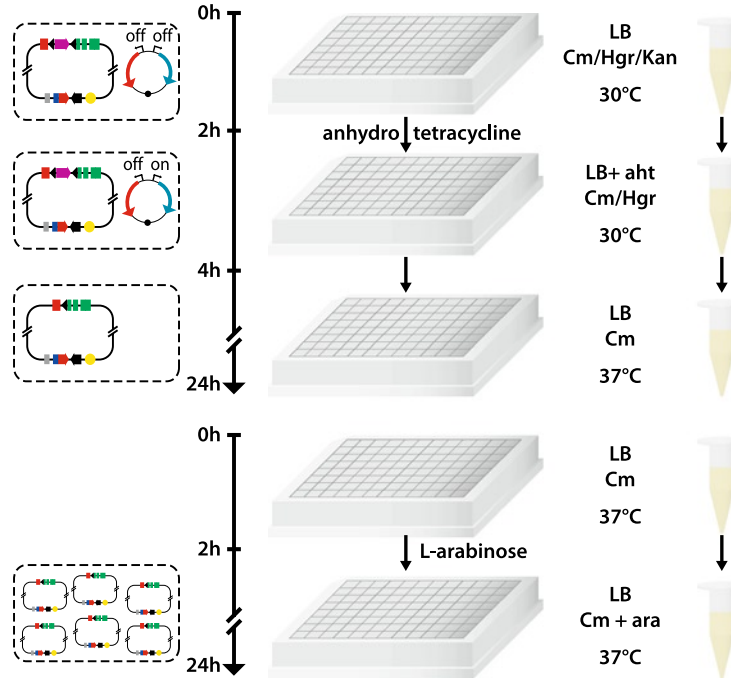

Fig. 5. Removal of selectable marker.

2. Seal the plate with an air-permeable seal and culture for 2 h at 30 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.
3. Use 100 µl of the cultures to inoculate 1 ml of LB + Cm per well/tube.
4. Seal the plate with an air-permeable seal and culture overnight at 37 °C with vigorous shaking. If using a single tube, puncture the lid with a sterile needle.

3.5. Verification of the Tagging Result

The recombinant clones are verified by sequencing (Fig. 6).

1. Design a pair of sequencing primers extending from within the tag toward the gene–tag junctions. If the recombineering cassette contains the FRT-flanked selectable marker, include the primer on the selectable cassette side before the first FRT.
2. Isolate the BAC/fosmid DNA from the modified clones. We suggest using the high-throughput miniprep protocol (see Chapter 25) or the Epicentre miniprep kit.
3. Sequence the modified clones using designed primers.
4. Verify the sequencing results. The sequencing reads should include the tag end-sequences and the homology arms. The FRT-flanked selectable marker should not be present.

3.6. DNA Purification and Fly Transformation

Fosmid DNA purified from the modified FlyFos clones can be directly used for fly transgenesis. l-Arabinose (Ara) is used to induce fosmids to high copy by activating oriV. This part of the protocol requires the Plasmid Maxi Kit (QIAGEN).

1. Inoculate 50 ml of LB + Cm with a single colony of FlyFos strain. Culture overnight at 37 °C with vigorous shaking.
2. Use 2 ×5 ml to inoculate 2 ×500 ml LB + Cm + Ara in 2,500 ml flasks. Culture overnight at 37 °C. Shake cultures vigorously.
3. Harvest the bacterial cells by centrifugation at 6,000× *g* for 15 min at 4 °C.
4. Resuspend the bacterial pellet from both flasks combined in 50 ml of Buffer P1.

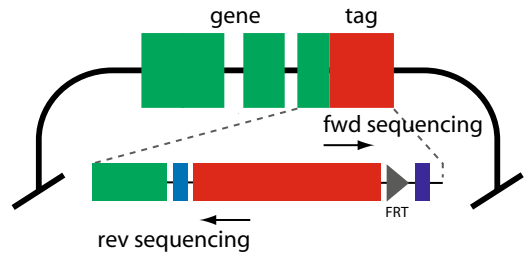

Fig. 6. Tagging verification.

5. Add 50 ml of Buffer P2, mix thoroughly by vigorously inverting 4–6 times, and incubate at room temperature for 5 min.
6. Add 50 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 30 min.
7. Centrifuge at ≥20,000× *g* for 30 min at 4 °C. Remove supernatant containing fosmid DNA promptly.
8. Place folded Whatmann filter in a 50-ml syringe. Prewet and compress filter by passing water through the syringe. Use such prepared syringe for filtering supernatant.
9. Precipitate the DNA by adding 105 ml (0.7 volumes) of room temperature isopropanol to the lysate. Centrifuge at ≥15,000× *g* for 30 min at 4 °C, and carefully decant the supernatant.
10. Redissolve the DNA pellet in 500 µl of warm (60 °C) TE buffer, pH 8.0, and add Buffer QBT to obtain a final volume of 12 ml.
11. Equilibrate a QIAGEN-tip 500 by applying 10 ml of Buffer QBT, and allow the column to empty by gravity flow.
12. Apply the DNA solution from step 10 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
13. Wash the QIAGEN-tip with 2 × 30 ml of Buffer QC.
14. Elute DNA with 15 ml of Buffer QF.
15. Precipitate DNA by adding 10.5 ml (0.7 volumes) of room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000× *g* for 30 min at 4 °C. Carefully decant the supernatant.
16. Wash DNA pellet with 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000× *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.
17. Wash DNA pellet again with 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000× *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.
18. Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume (250 µl) of warm (60 °C) nuclease-free water.
19. Use isolated fosmid DNA for injection into the *attP* landing line (see Note 15).

4. Notes

1. Use nuclease-free water to prepare DNA handling buffers and all enzymatic reactions.
2. Use Milli-Q or equivalent to minimize the salt concentration during electroporation.

3. Super Optimal broth with Catabolite repression (SOC) is a rich bacterial medium that greatly increases transformation efficiencies by facilitating cell survival after electroporation.
4. Yeast Extract, Nutrient Broth (YENB) medium is a rich, salt-free medium, optimal for preparation of the electrocompetent cells (10). While other media, like LB or SOC can be used for bacterial culture, two washing steps are required for optimal electroporation.
5. Active concentrations of hygromycin from different providers vary, test each batch to ensure that you are using the restrictive concentration and store it in single use aliquots at – 20°C.
6. The recombineering efficiency is proportional to the length of the homology arms and reverse-proportional to the length of the insert. Therefore, it might be wise to extend the homology arms for long recombineering cassettes. Moreover, as it is suggested that recombineering of fragments longer than 3 kb occurs in an alternate, low efficiency pathway (11), the recombineering cassette size should be kept below 3 kb if possible. Longer fragments can still be used for recombineering, however, lower efficiencies should be expected. Therefore, we recommend plating bacteria on selective agar (LA + Cm + Kan + Hgr, culture ON at 30°C) and screening clones by PCR after the tagging step.
7. We have developed and tested dozens of tagging cassettes that include various fluorescent markers. Some examples are presented in Fig. 7. The tagging cassettes are cloned in a pR6K-based (5) vector that can be propagated in *pir-116 E. coli* strain (Epicentre) only. This greatly reduces background originating from trace amounts of the PCR template that transforms bacteria (6, 8). If other vectors are used for amplification of the tagging cassette, we suggest digesting the PCR product with *DpnI* (NEB), that cuts methylated DNA only.
8. Minimize the amount of PCR cycles to avoid point mutations introduced by the polymerase. Increase the amount of PCR template if necessary. Use a good proof-reading polymerase (we recommend Phusion from NEB/Finnzymes).
9. We recommend using a PCR purification kit with oligonucleotide cut-off over 100 bases, such as Invitrogen PureLink™ PCR purification Kit.
10. When preparing electrocompetent cells, work in a cold room. Avoid exposing cells to temperatures higher than 41°C. For best efficiencies work as fast as possible.
11. The presented voltage (2,500 V) applies to cuvettes with 2 mm electrode gap. For other types of cuvettes, use 1,250 V/mm.

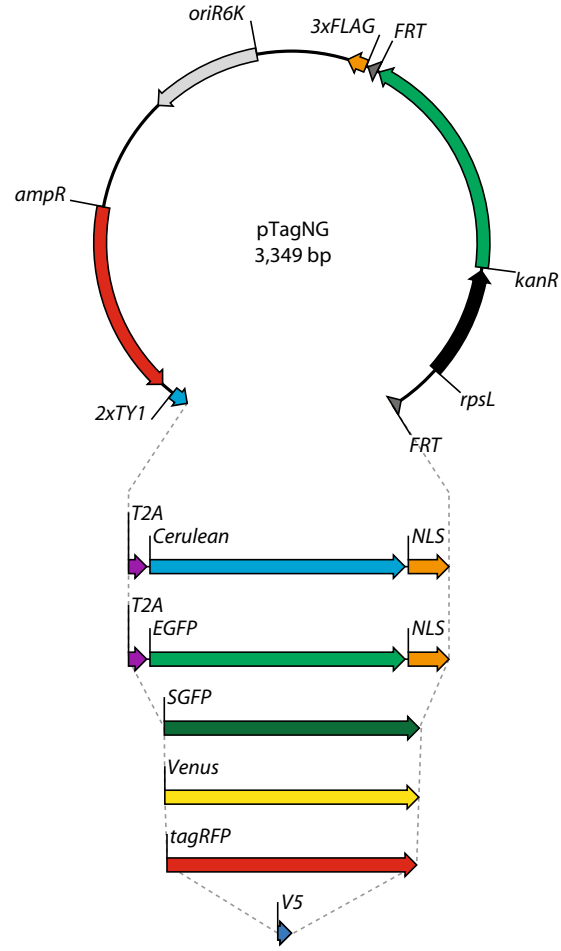

Fig. 7. Example recombineering vectors.

12. The pRedFlp plasmid (6) has a temperature-sensitive origin of replication from pSC101 (1). It has to be maintained at 30°C and can be removed from the cells by overnight growth at 37°C in the absence of hygromycin.
13. Plating on selective agar (LA + Cm + Kan) after the tagging step will give you an estimate of the success rate of recombineering. Expect between 10 and 100 cfu/reaction.
14. In our experiments, we have repeatedly obtained recombineering efficiencies (successfully validated out of attempted constructs) between 75 and 100%.
15. We strongly recommend using landing lines that express φC31 integrase in the posterior pole, such as *nanos* or *vasa* promoter-driven constructs (12). For best results, use the prepared DNA as soon as possible. Avoid freezing isolated fosmids.

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