Recombineering pipeline for tagging FlyFos clones

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Introduction

Before you start with the protocol, design the recombineering primers. They should include 50bp homology arms targeting the tagging site and 21 first bases of the tag region to amplify. Please take care of orientation of homology arms. All volumes are PER WELL of a 96-well plate.

Procedure

Day 0 – Preparation of PCR cassettes for tagging

1. Set up 50 µl PCR reactions in a 96-well plate format. Use of proofreading polymerase is recommended. Use high quality synthetic oligonucleotides, at least HPLC purified. The optimal annealing temperature is 57-63ºC. Do not use more cycles than necessary (20-25) to reduce the risk of PCR introduced mutations. Increase the amount of template if necessary to reduce the number of cycles.
2. Purify the products using Eppendorf Perfectprep PCR Cleanup 96.
3. Elute in 500 µl of distilled water (to final concentration of about 5 ng/µl).

Day 1 – Transformation of pRedFlp4

1. Pipette 1 ml of LB plus 12.5 µg/ml chloramphenicol to each 96-well.
2. Inoculate with 40 µl overnight culture of the FlyFos clones.
3. Seal the plate with breathable plate seal and grow for 2h at 37°C, 900 rpm.
4. Spin down for 10 min at 5000 g.
5. Discard the supernatant and press the plate onto a paper stack (made of 5-6 layers of clean paper tissue).
6. Add 1 ml of ice-cold sterile 10% glycerol to each plate.
7. Seal the plate with plastic seal and shake at 1400 rpm for 1 min.
8. Spin down for 10 min at 5000 g.
9. Discard the supernatant and press the plate onto a paper stack (made of 5-6 layers of clean paper tissue).
10. Add 100 µl of pRedFlp4 in H_2O (0.1 ng/µl).

11. Pipette up and down 5 times to suspend the pellet and transfer to a 96-well electroporation cuvette (BTX Harvard Apparatus 45-0450).
12. Electroporate at 2500 V.
13. Transfer the cells to a new plate with 1 ml of SOC medium per well.
14. Seal with the breathable film and let the cells recover for 1h at 30°C, 900 rpm.
15. Transfer 100 µl to a new plate with 1 ml of LB plus 12.5 µg/ml chloramphenicol and 20 µg/ml hygromycin.
16. Seal with breathable seal film and incubate overnight at 30°C, 900 rpm.

Day 2 – Tagging by Red/ET recombination

1. Pipette 1 ml of LB plus 12.5 µg/ml chloramphenicol and 20 µg/ml hygromycin to each 96-well.
2. Inoculate with 40 µl overnight culture of the fosmid plus pRedFlp4.
3. Seal the plate with breathable plate seal and grow for 2h at 30°C, 900 rpm.
4. Add 20 µl of 25% L-rhamnose to each well, seal again with breathable seal and grow for further 1h at 37°C, 900 rpm.
5. Spin down for 10 min at 5000 g.
6. Discard the supernatant and press the plate onto a paper stack (made of 5-6 layers of clean paper tissue).
7. Add 1.2 ml of cold sterile 10% glycerol to each plate.
8. Seal the plate with plastic seal and shake at 1400 rpm for 1 min.
9. Spin down for 10 min at 5000 g.
10. Discard the supernatant and press the plate onto a paper stack (made of 5-6 layers of clean paper tissue).
11. Add 100 µl of PCR product in H_2O (5 ng/µl).
12. Pipette up and down 5 times to suspend the pellet and transfer to the 96-well

electroporation cuvette.

13. Electroporate at 2500 V.
14. Transfer the cells to new plate with 1 ml of SOC medium per well.
15. Seal with the breathable film and let the cells recover for 1h at 30°C, 900 rpm.
16. Transfer 100 µl to a new plate with 1ml of LB plus 12.5 µg/ml chloramphenicol, 20 µg/ml hygromycin and 15 µg/ml kanamycin.
17. Seal with the breathable film and incubate overnight at 30°C, 900 rpm.

Day 3 – Removal of the kanamycin resistance gene

1. Pipette 1 ml of LB plus 12.5 µg/ml chloramphenicol, 20 µg/ml hygromycin and 200 nM anhydrotetracycline to each 96-well.
2. Inoculate with 10 µl overnight culture of the tagged fosmid.
3. Seal the plate with breathable plate seal and grow overnight at 37°C, 900 rpm.
4. The next day transfer 200 µl of the overnight culture to each of 2 plates with 800 µl of LB plus 25% glycerol, 12.5 µg/ml chloramphenicol.
5. Seal the plates, label them and store in 2 separate freezers.

A toolkit for high-throughput, cross-species gene engineering in Drosophila

by Radoslaw K Ejsmont, Mihail Sarov, Sylke Winkler, +1
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