pRed/ET mediated point mutation protocol

Gothenburg iGEM-team 2016

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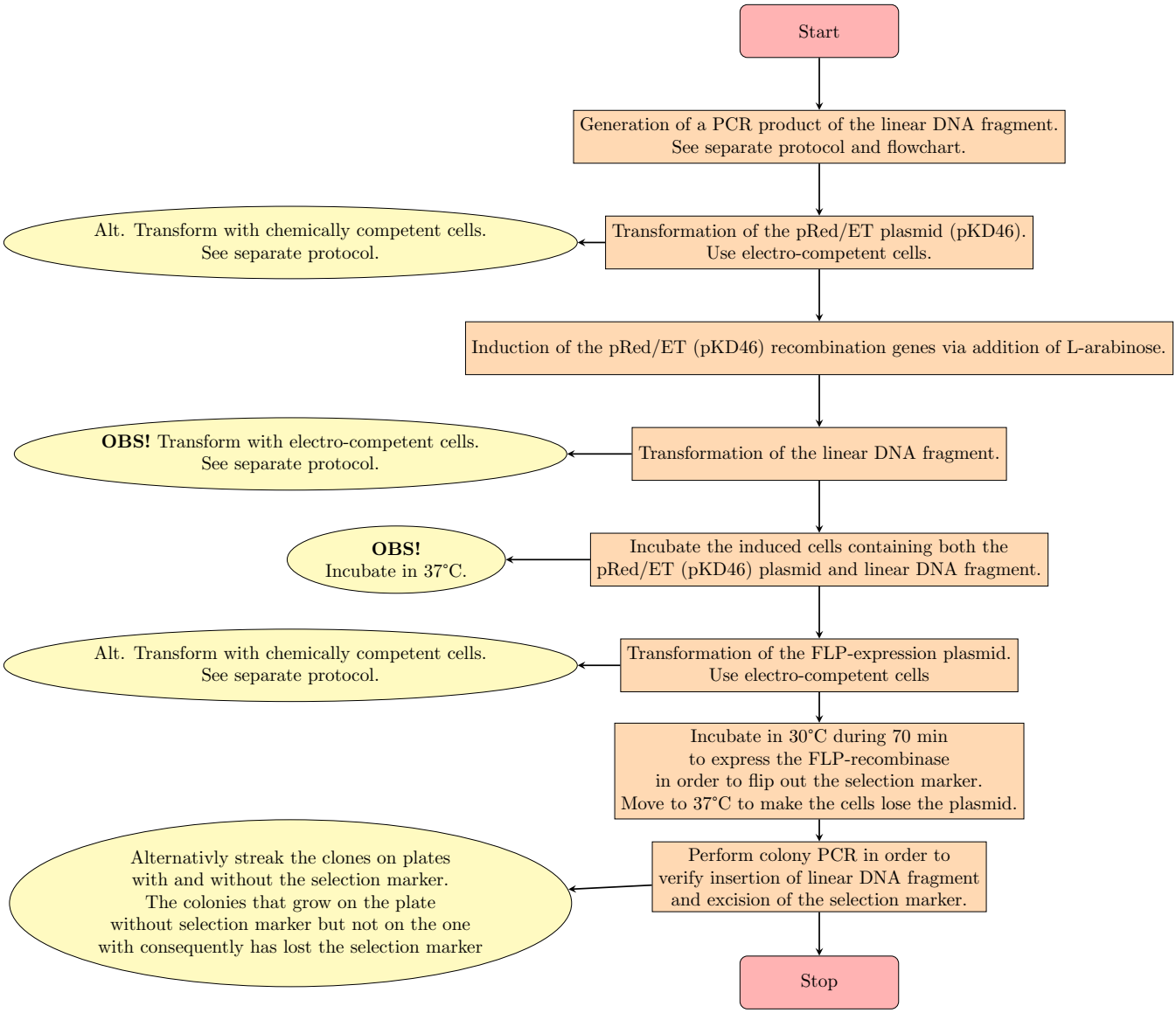
Experimental outline

Make note that the steps in the described procedure are gathered from "Quick and Easy E. coli Gene Deletion Kit" (Gene Bridges, Heidelberg) and adapted for our specific purposes and plasmids.



L-arabinose stock solution

Use 10% L-arabinose (Sigma A-3256) in ddH2O, fresh or frozen in small aliquots at -20°C. Use 50 µl stock solution per 1.4 ml LB for induction of recombination protein expression from pRedET. Frozen aliquots should not undergo more than three freeze-thaw cycles.

Media for antibiotic selection

Stock solutions should be stored at -20^{o}C. For selective LB medium, the antibiotic is dissolved in LB medium to the indicated working concentration:

- Chloramphenicol stock solution c = 30 mg/ml dissolved in ethanol.
  Working concentration 50 mg/ml.
- Ampicillin stock solution c = 100 mg/ml dissolved in 50% ethanol.
  Working concentration 50 mg/ml.
- Tetracycline stock solution c = 10 mg/ml dissolved in 75% ethanol.
  Working concentration for pRed/ET (pKD46) is 3 mg/ml. Tetracycline is light sensitive.
- Kanamycin stock solution c = 30 mg/ml dissolved in ddH20.
  Working concentration 15 mg/ml.

Selective LB plates are made by adding 15 g agar to 1 L LB medium. After boiling, cool to approx. 50°C, add the required antibiotics to yield the appropriate working concentrations and pour into petri dishes.

PCR

- Follow the specific instructions for the Phusion polymerase and use the calculated annealing temperature found in separate document.
- Verify product on gel.
- Purify the PCR product from the gel.

Transformation of the pRed/ET (pKD36) plasmid

Make an liquid o/n culture with LB medium. Preferably restreak colonies once or twice to achivie singular colonies. Incubate in 37°C.

Before starting make sure you have:

- Chilled ddH_2O (or 10% glycerol)
- Chilled electroporation cuvettes
- Cool benchtop centrifuge to 2°C

To make electro-competent cells and transform them

1. Set up one or two microfuge tubes containing fresh 1.4 ml LB medium and inoculate with 30 µl of fresh overnight culture.
2. Culture for 2-3 h at 37°C, shaking at 1000 rpm.
3. Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (at 2°C). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH2O (or 10% glycerol), pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 µl will be left in the tube with the pellet. Resuspend cells in the remainding liquid and keep the tube on ice.
4. Take the pRed/ET (pKD46) plasmid. Add 1 µl (20 µg) to your cell pellet. Mix briefly. Keep the tube on ice. Transfer the cell suspension from the tube to the chilled electroporation cuvette.

5. Electroporate at 1350 V, 10mF, 600 Ohms, a 5 ms pulse are recommended.

6. Resuspend in 1 ml LB and incubate in 30°C for 70 min while shaking. Plate on appropriate marker and incubate in 30°C o/n. Make sure to plate a control.

Induction of the pRed/ET plasmid and transformation of linear DNA

Inoculate a o/n liquid culture with LB medium from the plates achievied in the previous section.
Make the same preparations as in the prevous section.

1. Set up 4 microfuge tubes (2 for your own experiment and 2 for control experiment) containing 1.4 ml fresh LB medium conditioned with the same antibiotics as before. Inoculate two of them with 30 µl fresh overnight culture for your experiment, the other two with 30 µl of the overnight culture from the control. Incubate the tubes at 30°C for approximately 2 h, shaking at 1100 rpm until $OD_600 = 0.3$.

2. Add L-arabinose to the tubes giving a final concentration of 0.3%-0.4% w/v. This will induce the expression of the Red/ET (pKD46) recombination proteins. Leave the other tubes without induction as negative controls. Incubate all at 37°C, shaking for 45 min to 1 h.

3. Repeat step 3 from previous section.

4. Add 1-2 µl (200-400 ng) of your prepared linear DNA fragment to each of the two microfuge tubes (induced and uninduced), and pipette the mixture into the chilled electroporation cuvettes. In parallel, pipette 2 µl (400 ng) of linear DNA fragment into each of the two tubes of the control.

5. Repeat step 5 and 6 from previous section. Incubate in 37°C for 3 h instead. Incubate in 37°C o/n.

Induction of pRed/ET (pKD46) plasmid

OBS! Do not use D-arabinose. **OBS!**

Note: It is important that cells are incubated at 37°C, the temperature at which all proteins necessary for the subsequent recombination are expressed. There are about 5 copies of this temperature-sensitive plasmid per cell, and during one hour there is approximately 1 doubling step, meaning any daughter cell will still have on average 2-3 copies left and will also go on expressing the recombination proteins. The plasmid is actually lost after electroporation and recombination, that will say after transformation of the FLP-plasmid (pCP20) when cells are incubated at 37°C over night.

Removal of the selection marker with FRT/FLP-system

Repeat the procedure from the "Transformation of the pRed/ET (pKD36) plasmid" section. Use the plasmid containing the FLP-recombinase (pCP20) in the transformation step.

After incubating the plates o/n pick colonies for verification. Do a colony-PCR as well as streak the separate colonies on plates with the selection marker and on plates without. The colonies that only survive on the plate without selection marker has sucessfully flipped out the FRT-flanked selection marker from the cromosome.