GENE KNOCK-OUTS BY RECOMBINEERING in E. coli

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INTRODUCTION

The bacterial chromosome can be engineered in vivo by homologous recombination. Using PCR products or oligonucleotides as substrates, bacteriophage-encoded recombination functions can efficiently recombine sequences with short homologies (35-50 bp). This technology, termed recombineering, allows DNA to be inserted or deleted from the chromosome without regard to the presence or location or restriction sites. In the DY330, the prophage containing strain, the phage recombination functions are under the control of the bacteriophage λ temperature-sensitive CI857 repressor. At low temperatures (30-34 °C) the genes are repressed, upon a shift to 42°C, the genes are expressed at high levels. After temperature induction, the modifying DNA is introduced into DY330 by electroporation and recombinants obtained by screening for the designed selection. The engineered alleles can then be moved into any host strain of choice by P1 transduction. The protocol for generating gene knock-outs using DY330 expressing the defective lambdoid prophage recombination system λ red is described below.

MATERIALS

Recombineering strain DY330 W3110 ΔlacU169 gal490 pgIΔ8 λcI857 Δ(cro-bioA)

NOTE: Strain is biotin auxotroph and will not grow in minimal medium unless biotin is added (0.001% w/v final concentration).

MEDIA

- LB medium per litre:
  - Tryptone 10 g
  - Yeast Extract 5 g
  - NaCl 5 g
  - For solid media add 14 g Agar per litre
  - For selective media add the following concentrations of antibiotics:
### Antibiotic Stock Conc. (mg.ml⁻¹)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock Conc. (mg.ml⁻¹)</th>
<th>Solvent</th>
<th>Dilution</th>
<th>Final Conc. (µg.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>2.5</td>
<td>dH₂O</td>
<td>100X</td>
<td>25</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>25</td>
<td>dH₂O</td>
<td>1000X</td>
<td>2.5</td>
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<tr>
<td>Chloramphenicol</td>
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<td>EtOH</td>
<td>1000X</td>
<td>1.25</td>
</tr>
<tr>
<td>Apramycin</td>
<td>60</td>
<td>dH₂O</td>
<td>1000X</td>
<td>6</td>
</tr>
</tbody>
</table>

All antibiotics are supplied by Sigma-Aldrich Company Ltd. (Poole, Dorset) and filter sterilised before use.

### REAGENTS
- Tryptone (Oxoid Ltd. Basingstoke, Hampshire)
- Yeast Extract (Oxoid Ltd. Basingstoke, Hampshire)
- NaCl (Oxoid Ltd. Basingstoke, Hampshire)
- Agar (Oxoid Ltd. Basingstoke, Hampshire)
- Ice-cold Millipore water (MQ)
- M9 salts

### REAGENT SET-UP
- **M9 salts** prepared as stock solution per litre:
  - Anhydrous Na₂HPO₄ 6 g
  - Anhydrous KH₂PO₄ 3 g
  - NaCl 0.5 g
  - NH₄Cl 1 g
- Millipore water (MQ)– autoclave prior to use and chilled on ice before use

### EQUIPMENT
- 250 ml baffled flasks
- 32 °C Incubator (New Brunswick Scientific Co. Ltd. G-25 Incubator Shaker set to 200 rpm)
- 42°C Water bath
- Gene Pulsar II Electroporation conditions: 200 ohms, 25 µF, 1.8 kV
- Micro-centrifuge (Jouan A14 ultracentrifuge, 14000 rpm)
- Jouan MR22i centrifuge, SWM180.5 rotor
- 0.1 cm pathlength electroporation cuvettes (Bio-Rad Gene Pulsar Cat. No. 1652089)
PROCEDURE

TIMING ~ 6h. These conditions produce enough competent cells for 4 electroporations, scale up as required.

1. Grow a 5ml LB medium culture of DY330 overnight at 32°C, shaking at 200 rpm.
2. Dilute the overnight culture 1/70 into 35 ml LB medium in a 250 ml baffled flask, pre-warmed to 32°C. (0.5 ml overnight culture into 35ml LB). Incubate culture at 32°C 200 rpm until it reaches OD_{600} 0.4-0.6.
3. Transfer half the culture volume (usually 15ml) to a second, empty 250 ml baffled flask pre-warmed to 42°C (B) and incubate at 42°C for 10 min in a shaking water bath (200 rpm). The first, control flask (A) remains at 32°C.
4. Immediately after the temperature induction, the control (A) and induced (B) flasks are transferred to ice water for 10 minutes (occasional gentle swirling).
5. Both cultures are transferred to chilled 50ml Falcon tubes and the cells harvested by centrifugation for 9 minutes at 4°C, 4400 rpm. Discard supernatant.
6. Resuspend the pellet in 1 ml ice-cold MQ by pipetting up and down very gently. Keep the Falcon tube on ice whilst resuspending the pellet. Add a further 30ml of ice-cold MQ water and mix gently by inversion.
7. Harvest cells by centrifugation for 9 minutes at 4°C 4400 rpm. At this point the cells are very sensitive so handle carefully.
8. Discard the supernatant by carefully decanting and resuspend pellet in 1ml ice-cold MQ water by pipetting. Again, keep cells chilled by keeping Falcon tube on ice whilst resuspending.
9. Transfer cells to chilled 1.7ml microcentrifuge tubes. Spin cells down for 30 seconds at 14000 rpm, 4°C in a microcentrifuge.
10. Discard the supernatant then resuspend pellet in 200µl ice-cold MQ water by pipetting. At this point you should have 2 tubes: induced and uninduced cells in 200µl MQ water. Cells can be kept on ice for 1-2 hours, however, efficiency decreases with time.
11. Mix 50µl of cell suspension with ~100-150 ng of DNA in a chilled eppendorf. Transfer the Cell/DNA mixture to chilled 0.1 cm pathlength electroporation cuvettes

Note: Virtually salt free DNA is required for electroporation and this needs to be tested before performing the experiment using the electroporator. Cleaning up the DNA PCR product with the Qiagen PCR clean-up kit is usually sufficient but may need to be performed twice. To test DNA use water instead of cell suspension (see step 12).

Your negative controls are:
1. Induced cells with no DNA added.
2. Uninduced cells with DNA added.

12. Electroporation conditions: 200 ohms, 25 µF, 1.8 kV. The 200 µl induced cell suspension is enough for 4 electroporations.
Note: Ideally, the time constant for electroporation should be between 4.7-4.9 ms. Time constants less than this could be due to high salt content in the DNA solution or due to insufficient washing of cells. dH₂O should give you a time constant of 5ms.

13. Immediately after electroporation add 1ml of room temperature LB to the cuvette. This is your neat cell suspension.

14. **RECOVERY and PLATING OF CELLS.** There are two ways of doing this:

**Nitrocellulose membrane method (more efficient).**
- Following electroporation transfer neat cell suspension into eppendorf tube.
- Serially dilute the neat suspension to 10⁶ using M9 salts.
- Place a nitrocellulose membrane (autoclaved in aluminium foil) onto an LB plate prewarmed to 32°C.
- Plate 100µl of the neat suspension and each dilution onto the nitrocellulose membrane/LB agar plate. Incubate at 32°C for 3 hours.
- For negative controls only plate neat suspension.
- Remove the membrane from the LB plate using sterile forceps and place onto selective LB agar plates. Incubate at 32 °C overnight.
- In addition, plate 100µl of dilutions 10⁻⁵ and 10⁻⁶ onto non-selective LB agar plates to check viability of cells after electroporation. Incubate at 32 °C overnight.

**Note:** Recovery of cell seems to be better when the cell suspension is less dense and this method is to be preferred over the Direct plating method described below.

**Direct plating method:**
- Following electroporation transfer neat cell suspension into eppendorf tube.
- Recover cells at 32°C for 2 hours shaking (200 rpm). Taping the tubes to a rack usually works fine.
- Serially dilute the neat suspension to 10⁶ using M9 salts (100µl of neat cell suspension to 900µl M9 →10⁻¹).
- Plate 100µl of the neat suspension and each of the dilutions onto selective LB agar plates. Incubate overnight at 32°C.
- For negative controls only plate neat suspension.
- In addition, plate 100 µl of dilutions 10⁻⁵ and 10⁻⁶ onto non-selective LB agar plates to check viability of cells after electroporation. Incubate at 32 °C overnight.

**References**