GENE KNOCK-INS AT THE LACZ/Y LOCUS BY RECOMBINEERING in *E. coli*

CONTACTS

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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 DY378 (W3110 *λcI857 Δ(cro-bioA)*), 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 DY378 by electroporation and recombinants obtained by screening for the designed selection. This technology was used to create MJF618, a DY378 derivative in which a non-essential gene upstream of the Lac operon, *cynX*, has been knocked-out and replaced with an apramycin resistance gene. This strain allows us to make targeted gene knock-in mutants at the lacZ/Y locus marked by apramycin resistance. The engineered alleles can then be moved into any host strain of choice by P1 transduction. The protocol for generating gene knock-ins using MJF618 expressing the defective lambdoid prophage recombination system λ red is described below.

MATERIALS

Recombineering strain MJF618 (DY378 <*cynX::Apr*>)

NOTE: Strains are 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. ($mg.ml^{-1}$)**	**Solvent**	**Dilution**	**Final Conc. ($\mu g.ml^{-1}$)**
Ampicilin	2.5	dH_2O	100X	25
Kanamycin	25	dH_2O	1000X	2.5
Chloramphenicol	12.5	EtOH	1000X	1.25
Apramycin	60	dH_2O	1000X	6

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

REAGENTS

- Tryptone (Oxoid Ltd. Basingstoke, Hampshire)
- Yeast Extract (Oxoid Ltd. Basingstoke, Hampshire)
- NaCl (Oxoid Ltd. Basingstoke, Hampshire)
- Agar (Oxoid Ltd. Basingstoke, Hampshire)
- DMF (Sigma-Aldrich Company Ltd. Poole, Dorset)
- 160 $mg.ml^{-1}$ X-gal (Melford)
- 40 $mg.ml^{-1}$ IPTG (Sigma-Aldrich Company Ltd. Poole, Dorset)
- Ice-cold Millipore water (MQ)
- M9 salts

REAGENT SET-UP

- **M9 salts** prepared as stock solution per litre dH_2O and autoclaved prior to use:
  - Anhydrous Na_2HPO_4 6 g
  - Anhydrous KH_2PO_4 3 g
  - NaCl 0.5 g
  - NH_4Cl 1g

- Millipore water (MQ)– autoclave prior to use and chill on ice.

- **X-gal**: Prepare a 160 mg.ml^{-1} stock of X-gal by weighing 0.16 g into an eppendorf and dissolving in 1 ml DMF before filer sterilizing using a 0.45 μm Whatman filter. This is a X1000 stock solution.
- **IPTG**: Prepare a 40 mg.ml^{-1} stock of IIPTG by weighing 0.4 g IPTG into a sterilin and dissolving in 10 ml dH_2O before filer sterilizing using a 0.45 μm Whatman filter. This is a X1000 stock solution.
- **LB X-gal/IPTG plates:** for 15 plates: Autoclave 400 ml LB agar (4 g Tryptone, 2 g Yeast extract, 2 g NaCl, 5.6 g Agar in 400 ml dH_2O) and allow to cool to <60°C before adding 400 μl of the X-gal and IPTG stock solutions, stir well and pour plates. These plates will keep for one week in a cold room wrapped in foil.

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

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

1. Inoculate a sterilin containing 5 ml LB medium with a single colony of MJF618
2. Grow culture overnight at 32°C, shaking at 200 rpm.
3. 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.
4. Transfer half the culture volume (usually 15 ml) 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.
5. Immediately after the temperature induction, the control (A) and induced (B) flasks are transferred to ice water for 10 minutes (occasional gentle swirling).
6. 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.
7. Resuspend the pellet in 1 ml ice-cold MQ by pipetting up and down very gently. Keep the Falcon tube is kept on ice whilst resuspending the pellet. Add a further 30ml of ice-cold MQ water and mix gently by inversion.
8. Harvest cells by centrifugation for 9 minutes at 4°C 4400 rpm. At this point the cells are very sensitive so handle carefully.

9. 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.
10. Transfer cells to chilled 1.7ml microcentrifuge. Spin cells down for 30 seconds at 14000 rpm, 4°C in a microcentrifuge.
11. 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.
12. 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.

13. 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. dH_2O should give you a time constant of 5ms.

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

15. **RECOVERY and PLATING OF CELLS**
    - 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 10^{-6} using M9 salts (100µl of neat cell suspension to 900µl M9 $\rightarrow 10^{-1}$).
    - Plate 100µl of the 10^{-3} to 10^{-6} dilutions onto LB X-gal/IPTG plates. Incubate overnight at 32°C. The aim is to select white colonies from the dilution in which the colonies are well separated. To increase your chance of getting several putative white knock-in recombinants plating several 100 µl aliquots of each dilution onto LB X-gal/IPTG plates will give more colonies for blue/white screening.

- 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.
- Putative white recombinants should be streaked out at least twice on LB X-gal/IPTG plates to ensure they were single genetically identical white colonies. Knock-ins are then verified by PCR.

References

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G. and Court, D.L. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A* **97**:5978-5983