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EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: *Marker-less multiple gene deletions in P. putida KT2440*

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Instruction

Marker-less multiple gene deletions in P. putida KT2440

1. Introduction / Purpose

In this protocol a method is described to make multiple gene-deletion in *P. putida* KT2440. The protocol is an adaptation for *P. putida* of the method described by Fehér *et al.* (for *E. coli*). The two plasmids described here, pSW(I-Sce-I) and pEMG, can be delivered into the cells by electroporation or tri-parental mating (using an *E. coli* helper strain that harbors the pRK600 plasmid).

For this deletion process the I-SceI expressing plasmid (pSW) could be introduced before (preferred option if you plan to do multiple deletions within the same strain) or after obtaining your co-integrate.
The second will be described here.

Keywords: Gene deletion – marker-less – KT2440 – *P. putida* – knock-out

2. Equipment and chemicals

2.1. Equipment

- Centrifuge for eppendorf tubes
- Electroporation machine
- PCR machine

2.2. Chemicals

- PCR purification kit
- PCR reagents
- 300 mM sucrose
- 1-2mM 3-methylbenzoate

2.3. Bacterial strains

Pseudomonas putida KT2440, *E.coli* Dh5αλpir

2.4. Other materials

- Plasmid pSW(I-Sce-I)
- Plasmid pEMG or pGNW
- LB plates with kanamycin, x-gal, and iptg
- LB plates with kanamycin 50 µg ml^{-1} and ampicillin 600 µg ml^{-1}
- LB plates with ampicillin 600 µg ml^{-1}
- LB plates with ampicillin 500 µg ml^{-1}
- Primers outside of TS1 and TS2
- *M13 primers: fw=tgtaaaacgacggccagt and rev=caggaaacagctatgaccatg*

3. Media and Buffers

- LB medium

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4. Procedures

The current example explains the procedure for the deletion of aceEF operon (Pseudomonas putida KT2440).

Cloning region of interest into the suicide plasmid pEMG or pGNW

The cloning step could be performed by 2 methods: [1] PCR regions and cloning each region separately into the plasmid or [2] by overlap PCR, which is described here.

Overlap PCR

To construct the corresponding recombinogenic vectors, 500 bp (TS1) upstream and 500 bp downstream (TS2) regions of aceEF operon should be amplified separately and then join in a single DNA segment by overlap PCR (Fig. 1)

- Do the first PCR reactions to amplify the 500 bp upstream and downstream regions using a fresh colony of KT2440 as template.

- Prepare the second overlap PCR reaction using 1µl from each of the reaction products of the first PCR as template.

- Clean up the second PCR product using a purification column, digest with the appropriate restriction enzymes, inactivate them, and ligate to the suicide vector (pEMG or pGNW).

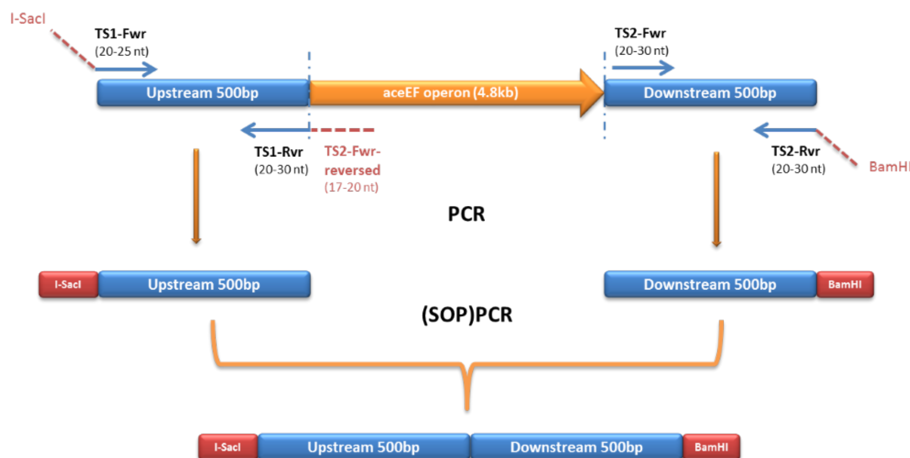

Figure 1

Transformation of E.coli *Dh5αλpir*

- Transform *E. coli* DH5αλpir with an aliquot of the ligation mixture and plate onto LB plates with kanamycin, X-Gal and IPTG.

- Select a few white colonies, re-streak them and check for the presence of the correct plasmid.
- Use M13 primers (few bases outside of the pGNW/pEMG polylinker) to select a positive clone and send it to sequence to confirm that the targeting sequences don't have sequence errors.

- **Note:** *Use I-SceI and the restriction profile of the constructed plasmid in the case that you use M13 primers for selection and sequencing. M13 bind just a few bases far from I-SceI sites of pEMG, so the sequencing cannot give you reliable results for that region*

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Cointegration of pEMG-aceEF(TS1-2)

Preparation of P. putida *KT2440 electrocompetent cells and introduction of pEMG-aceEF(TS1-2) or pGNW-aceEF(TS1-TS2)*

- Grow overnight a 20 ml culture of KT2440 in LB medium at 30 °C with shaking.
- Centrifuge at room temperature for 10 minutes.
- Remove supernatant and resuspend the pellet gently in 10 ml of 300 mM sucrose and centrifuge as in step 2.
- Discard supernatant, add 1 ml of 300 mM sucrose, resuspend the pellet and transfer it to an eppendorf tube.
- Centrifuge at 12000 rpm for 2 minutes at room temperature.
- Dispose of supernatant and add 500 µl of 300 mM sucrose, resuspend and distribute in 100 µl aliquots.
- Add 300-400 ng of your construct plasmid to a 100 µl aliquot of KT2440, mix gently and transfer to a 2 mm gap width electroporation cuvette and proceed to electroporate.
- Add 1 ml of LB or SOC and incubate 2 hours at 30 °C with shaking.
- Plate everything onto LB+kanamycin 50 µg ml^{-1} and incubate the plates for 2 days. (After 2 days there are a few big colonies which probably contain 2 population, the Km^r and Km^s)
- Streak a few big colonies and use colony PCR to check for co-integration events.

  Note: *For the selection of the right co-integrates, design and use primers which bind outside of the TS1-gene-TS2 region. In this way you are looking just for a single large PCR product instead of selecting with TS1-Fwr, TS2-Rvr. In the case of aceEF the desired product had the size of 10kb (Fig. 2 and Fig. 3).*

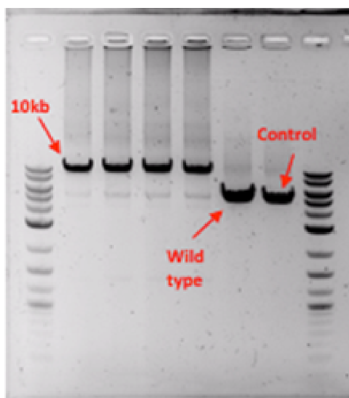

Figure 2

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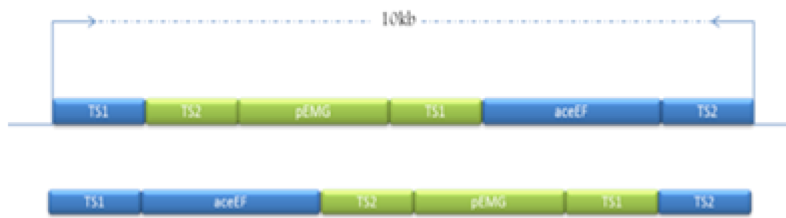

Figure 3

Delivery of pSW (I-SceI)

- Prepare electrocompetent cells of your co-integrate as described before.
- The pSW(I-SceI) plasmid described here bears the ampicillin resistance gene.
- Add 50 ng of pSW(I-SceI) plasmid to a 100 µl aliquot of your cointegrate, mix gently and transfer to a 2 mm gap width electroporation cuvette and proceed to electroporate.
- Add 1 ml of LB or SOC and incubate 1 hour at 30 °C with shaking.
- Plate several dilutions onto LB+Km50+ampicillin 600 µg ml^{-1} (KT2440 is naturally resistant to ampicillin but such concentration allows plasmid selection).
- Streak a few colonies and check the presence of the plasmid by miniprep or PCR (not essential step).

Induction of the I-SceI enzyme

- Start by diluting overnight cells to an OD600 of 0.1 and induce with 1-2mM 3-methylbenzoate for 6-7 h.
- Plate a few dilutions onto LB or LB plus Amp600 (if you wish to maintain the pSW-I plasmid for further deletions).
- Restreak colonies in LB and LB+Km50 to check the loss of the co-integrated plasmid.
- Select a few kanamycin sensitive clones and differentiate by colony PCR between WT and deleted clones (the proportion is usually close to 50%). For the selection of the knockout colonies use the primers outside of TS1 and TS2. The knockout colonies should give a size product of the size of TS1-TS2 (col 5-6) and the wild type the normal control size (col 1, 2, 3, 4) (Fig. 4).

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Figure 4

Curation of pSW(I-SceI)

- Pick a clone with deletion and do several passages in LB without antibiotics. In order to do that grow overnight in LB at 30 °C with shaking, then transfer to a new tube with fresh LB and repeat the process for 3 days.

- Plate onto LB appropriate dilutions to obtain separate colonies. Restreak several colonies on LB and LB+amp500.

- Select amp sensitive colonies and double check the plasmid curation by colony PCR using the primer pair described before.

5. *Remarks / troubleshooting*

- Use I-SceI and the restriction profile of the constructed plasmid in the case that you use M13 primers for selection and sequencing. M13 bind just a few bases far from I-SceI sites of pEMG, so the sequencing cannot give you reliable results for that region

- For the selection of the right co-integrates, design and use primers which bind outside of the TS1-gene-TS2 region. In this way you are looking just for a single large PCR product instead of selecting with TS1-Fwr, TS2-Rvr. In the case of aceEF the desired product had the size of 10kb.

6. *References*

Fehér T., Karcagi I., Győrfy Z., Umenhoffer K., Csörgő B., Pósfai G. (2008) *Scarless Engineering of the Escherichia coli Genome.* In: Osterman A.L., Gerdes S.Y. (eds) Microbial Gene Essentiality: Protocols and Bioinformatics. Methods in Molecular Biology™, vol 416. Humana Press

7. *Acknowledgements*

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