WCSB, Wageningen University	EPP-SOP-WU02 Version 1.1

EPP - Standard Operating Procedure

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

Title: Multiplex automated genome engineering (MAGE) for *Pseudomonas putida*

***In vivo* genome editing that uses synthetic ssDNA to introduce targeted modifications at multiple loci directly into the *Pseudomonas putida* chromosome in a cyclical manner for genome-wide editing.**

distribution list			
changes to prior version: added a figure for the procedure section			
	name	signature	date
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Instruction

Multiplex automated genome engineering (MAGE) for *Pseudomonas putida*

1 Introduction / Purpose

Multiplex automated genome engineering (MAGE) is a powerful technology which allows an investigator to create several independent or combinatorial genome modifications in a population of live cells.

MAGE harnesses phage homologous recombination proteins to generate targeted, fast, scarless modifications of bacterial chromosomes across many genomic loci. The recombinases used in MAGE act on synthetic ssDNA oligonucleotides introduced to a cell population which are designed with 5' – and 3' terminal homology arms that are complementary to target sequences in the chromosome. Oligos will anneal to their lagging strand of the target sequence during replication, and then they will be stably inherited after another round of replication. For a specific mutation, the oligo can skip, mispair or add bases respecting the target region, causing deletion, mismatch or insertion, respectively.

The technology is based on multiple cycles of recombineering using the *P. putida* characterized recombinase Rec2.

MAGE is aimed to genome-wide editing or for recoding projects that are not possible with other methods. In this case, our purpose is the recoding of the *P. putida* genome in which single nucleotide mutations have to be generated in hundreds of genes.

2 Equipment and chemicals

2.1 Equipment

- ⇨ Stationary incubator set at 30ºC
- ⇨ Shaking incubator set at 30ºC
- ⇨ Shaking water bath set at 42ºC
- ⇨ Ice bucket
- ⇨ Centrifuge kept at room temperature
- ⇨ Microcentrifuge kept at room temperature
- ⇨ Electroporator
- ⇨ Electroporation cuvettes with 1mm
- ⇨ Thermal cycler
- ⇨ PCR tubes
- ⇨ Sterile 96-well culture plates
- ⇨ Single-channel pipettes for 0.5-5000 µl volumes and sterile filter tips
- ⇨ Multichannel pipette for 0.5-200 µl volumes
- ⇨ Agarose gel electrophoresis apparatus
- ⇨ Sterile 1.7 ml microcentrifuge tubes
- ⇨ Sterile 50 ml culture tubes
- ⇨ Sterile Petri dishes
- ⇨ Inoculation hooks
- ⇨ Spectrophotometer
- ⇨ Spectrophotometer cuvettes

2.2 Chemicals

- User-defined synthetic ssDNA oligos for MAGE
- User-defined PCR primers for PCR
- Sterile distilled H_2O
- Tryptone
- Yeast extract
- Sodium chloride
- Agar
- Agarose
- DNA ladder
- Kanamycin
- Sucrose
- Nucleic acid stain: SYBR Safe

2.3. Bacterial strains

- *P. putida* strain: MAGE is performed in strains KT2440 or EM42 that carry the plasmid pSEVA251-cIpL-Rec2-mutL36K

2.4. Other materials

- Plasmid pSEVA251-cIpL-Rec2-mutL36K: contains a cIpL temperature system which induces the expression of the recombinase gene Rec2 and the mutL36K gene which prevents the action of the mismatch repair system. CIpL system is induced at 42ºC by inhibition of the repressor cI which is bounded to the pL promoter controlling the downstream genes at lower temperatures. The plasmid confers resistance to Kanamycin.
- User-defined synthetic ssDNA oligos for MAGE
- User-defined PCR primers for PCR

3. Media and buffers

3.1 LB (agar)/Kn_{50}*

- 10 g/l Tryptone
- 10 g/l NaCl
- 5 g/l Yeast Extract
- * 15 g/l Agar
- 50 ng/µl Kanamycin

3.2 TB/Kn_{50}

- 12 g/l Tryptone
- 24 g/l Yeast Extract
- 4 ml/l Glycerol
- 100 ml/l Phosphate buffer

3.3 Phosphate buffer

- 23.12 g/l KH_2PO_4
- 125.4 K_2HPO_4

3.4 Sucrose

- 300 mM Sucrose

4. Procedures

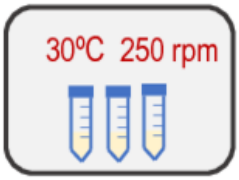
4.1 Cycle

1. Grow strain in LB/Kn_{50} in a 30ºC/250 rpm shaking incubator O/N.
2. Inoculate with the O/N culture 20 ml of fresh LB/Kn_{50} at DO=0.1. Grow at 30ºC/250 rpm until the cells reach mid-log phase, as measured by OD≈ 0.4-0.5 (approx. 2 h).
3. Induce the system by incubating at 42ºC for 5 min in a water bath with shaking.
4. Place culture in ice for 5 min.
5. Competent cell preparation: wash cells with 300 mM Sucrose (10 ml, 5 ml, 1 ml) centrifuging at 4500 rpm/10 min between washes at RT. Resuspend cells in 200 µl 300 mM Sucrose.
6. Electroporate a 100 µl aliquot with 1 µl of recombineering oligonucleotide 100 µM (≈ 3 µg). For mutagenesis of more than one target site, a complex oligo pool is created by mixing the oligos that target separate loci. Generally, the final total oligo concentration in a complex pool is held at 1-20 µM, with each constituent contributing an equal amount. To create a 50-µl equimolar pool of n oligos at 20 µM aggregate concentration, (50/n)-µl volumes of each 20 µM stock would be mixed.
7. Inoculate electroporated cells in 5 ml of fresh TB/Kn_{50} and let them recover for 1 h at 30ºC/250 rpm in a shaking incubator.
8. Add 15 ml LB/Kn_{50} to the inoculated 5 ml of TB/Kn_{50} and incubate until cells reach OD=0.5. Cells take around 5 h to reach this OD, so only one cycle can be performed per day. Consequently, after the addition of the 15 ml of LB/Kn_{50} it is recommended to incubate for at least 4 h at 30ºC/250 rpm in a shaking incubator and after that place the cells in the fridge O/N (4 ºC). Next day, cultures are reactivated again for 1-2 h at 30ºC/250 rpm in a shaking incubator until they reach the desired OD (0.5). It is important to recover electroporated cultures long enough to allow at least two complete cycles of genome replication.
9. Repeat from step 3.

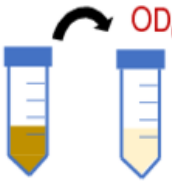
(A graphical work-flow for this procedure is shown below).

Protocol Recombineering *P. putida*

1 Grow liquid culture of *P. putida* O/N in LB/Kn$_{50}$ at 30ºC and 250 rpm.



2 OD$_{600}$ = 0.1



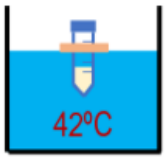
Use the O/N culture to inoculate a new 20 ml culture of fresh LB/Kn$_{50}$ at OD$_{600}$ = 0.1.

3 30ºC 250 rpm

OD$_{600}$ = 0.4-0.5

Grow at 30ºC and 250 rpm until the OD$_{600}$ reaches ≈ 0.5.

4 **Induction** of the system by incubating the culture at 42ºC for 5 minutes in the water bath with shaking.



5 min

5 ice 5 min

Immediately after, place the culture in ice for 5 minutes.

6

Competent cells preparation
Centrifuge the 20 ml culture for 10 minutes at 4700 rpm at RT, discard supernatant and resuspend in 10 ml 300 mM Sucrose.

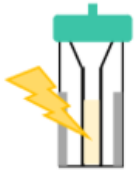
7

Centrifuge the 10 ml culture for 10 minutes at 4700 rpm at RT, discard supernatant, resuspend in 1 ml 300 mM Sucrose and transfer the volume to a 1.5 Eppendorf tube.

8 Centrifuge the 1 ml culture for 2 minutes at 12000 rpm at RT, discard supernatant and resuspend in 200 µl 300 mM Sucrose.

200 µl
300 mM
Sucrose

9



Electroporate a 100 µl aliquot with 1 µl of recombineering oligonucleotide 100 µM at 2500 V, 25 uF and 200 Ω.

10 30ºC 250 rpm

Inoculate a 5 ml fresh TB/Kn$_{50}$ culture with the electroporated cells for **cell recovery**. Incubate at 30ºC and 250 rpm for 1 h and then add 15 ml of fresh LB/Kn$_{50}$. Incubate O/N at 30ºC and 250 rpm.

11 Checking

Plate culture at different dilutions in LB agar. Incubate O/N at 30ºC. Count colonies and streak 10 of them in a new plate for checking.

12 Check allelic changes: amplification by PCR, sequencing...

11 Continuous cycles

After adding 15 ml of fresh LB/Kn$_{50}$ incubate the cells at 30ºC and 250 rpm for 4 h. Then keep the culture at 4ºC in the fridge O/N. Next day, grow the culture again at 30ºC and 250 rpm until the OD$_{600}$ reaches ≈ 0.5 like in Step 3 (approx. 1.5 h). Continue with Step 4.

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4.2 Checking of the mutations

1. Recover cells by incubating them at 30ºC/250 rpm in a shaking incubator O/N instead of performing steps 8 and 9.
2. Prepare serial tenfold dilutions for each MAGE sample.
3. Spread 100 µl of each dilution on plates selective for the mutagenized strain. Incubate the plates at 30ºC O/N until colonies appear.
4. By using a multichannel pipette, fill each well of a 96-well culture plate with 150 µl of LB.
5. Use pipette tips to pick colonies from each sample, and then transfer each colony to one well of the 96-well plate.
6. Grow the picked colonies at 30ºC/250 rpm in a shaking incubator until all wells have reached mid-log phase, as measured by the OD_{600}.
7. Set up two PCRs for each isolated colony (MASC-PCR). To supply template DNA, add 1 µl from each well of the 96-well plate to both WT and mutant reactions.
8. Visualize 4 µl of each PCR product on an 1.5% (wt/vol) agarose gel to determine the genotype of each colony.
9. Freeze and store the pure cultures for the colonies that screened correctly, and then confirm genotype with Sanger sequencing.

5. Remarks/troubleshooting

5.1 General remarks

This protocol is currently under optimization.

5.2 Troubleshooting

- **Arching during electroporation:** Caused by residual salt in either the cell pellet or the introduced DNA could be solved by washing the cells one more time with 300 mM Sucrose.
- **No outgrowth during recovery:** Caused by too many cells killed by electroporation could be solved by washing the cells one more time with 300 mM Sucrose.
- **No colonies on either experiment or control plates:** Caused by poor MAGE ARF could be solved by carrying out more MAGE cyles and by inducing cells at proper OD_{600}.
- **Colonies on both experiment and negative control plates:** Caused by not enough antibiotics could be solved by preparing new plates with appropriate amount of antibiotics. Caused by contamination could be solved by re-streaking strains or starting from collected cells from a previous cycle.
- **All PCR bands show the wild type:** Caused by poor MAGE allelic replacement frequency could be solved by additional MAGE cycles. Cause by inadequate screen could be solved by screening more colonies.

6. Biosafety

No biosafety issues were associated with this protocol when applied to *Pseudomonas putida.* The protocol was developed and performed at an MLI /BSLII laboratory. The cycle experiments are performed in the flow cabinet to prevent contamination.

7. Acknowledgements

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