Recombineering with Red®/ET®
Modification of the Bacterial Chromosome

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Metabolic engineering to design and construct microorganisms suitable for the production of industrial products like ethanol or aromatic amino acids requires the disruption of specific genes on the bacterial chromosome.

Regulatory circuits, the uptake of carbon and amino acids, the glycolytic and pentose phosphate pathway, as well as the common aromatic amino acid pathway have to be manipulated.

The complexity of the necessary modifications requires a tool allowing the precise knock-out or alteration of multiple genes without leaving antibiotic selection markers. Red/ET Recombination (1) also known as recombineering is an easy to use modification system for prokaryotic functional genomics.

There is proven evidence that Red/ET works not only in E.coli, but also in Salmonella, Shigella, Yersinia, Serratia and Citrobacter.

Here we demonstrate the easy and precise knock-out of the major mannose transporter (manXYZ) of E. coli strain DH10B using a FRT flanked kanamycin-resistance cassette. The selection marker was subsequently removed by a FLP-recombinase step.

Red/ET Recombination + FRT-neo-FRT cassette

Inactivation of $manX$ by insertion of a FRT-neo-cassette.

FRT-flanked cassette

Flp recombination + 705-FLP plasmid

Replacement of the selection marker by subsequent FLP-recombinase step, $manXYZ$ maintains inactivated.
Verification of the correct insertion site by PCR

PCR primer combinations 4/5 (lane 1), 4/6 (lane 2), 2/3 (lane 3) and 1/3 (lane 4) to confirm the correct insertion of the cassette by Red/ET recombination. After FLP recombination primer combination 2/5 (lane 5) amplifies the DNA fragment without the cassette.
Verification of the expected phenotype by physiological tests

Only cells from strain DH10B show the red phenotype on McConkey (Mannose) plates indicating mannose metabolism (left plate). The red phenotype of all three strains on the McConkey (Glucose) plates indicates a specific knock-out of the mannose transport system (right plate). While the intermediate strain (DH10B manX::FRTneo) grows on LB Kan plates, the final strain does not grow on kanamycin indicating a complete removal of the resistance marker (middle plate).
Strain DH10B shows a normal growth in both minimal medium plus glucose (DH10B MM-Glc; yellow) and minimal medium plus mannose (DH10B MM-Man; pale blue). The intermediate strain no longer grows on mannose (FRTneo MM-Man; violet). This phenotype remains, even after removal of the kanamycin cassette (FRT MM-Man; pink). The strain shows normal growth on glucose (FRT MM-Glc; dark blue).
Verification of specificity

*E.coli* cells from all three strains were embedded in agarose and digested with *ApaI* and *NotI*. DNA fragments were separated by Pulsed Field Gel Electrophoresis.

Due to an additional *NotI* site which flanks the FRT cassette, the 120kb *NotI* fragment on which the mannose transporter is located (see Heath et al. 1992) disappears after insertion of the FRT-flanked neomycin cassette while an additional 90kb fragment appears instead in lanes 5 and 6.

Beside this predicted difference in the *NotI* pattern, no other changes are visible, neither in the *ApaI* nor in the *NotI* digest indicating that no unintended rearrangements took place.

Lanes 1 + 4: DH10B
Lanes 2 + 5: DH10B manX::FRTneo
Lane 3 + 6: DH10B manX::FRT