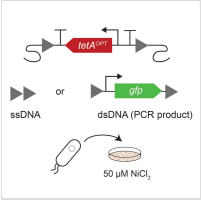

Oct 11, 2022

Gene knockout strategy

In 1 collection

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dx.doi.org/10.17504/protocols.io.n92ldzp2xv5b/v1

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ABSTRACT

This protocol collection describes how to use our optimised *tetA*^{OPT} dual selection marker in *E. coli* K12 and Nissle. This dual selection marker can be used for positive selection based on tetracycline resistance and counterselection based on $NiCl_2$ sensitivity. *tetA* can be used to engineer all stages of the central dogma of molecular biology. On the DNA-level *tetA*^{OPT} can be used to create scarless knockouts across the *E. coli* genome with an efficiency above 90%, whereas recombinant gene integrations can be achieved with approximately 50% efficiency. On the expression level, *tetA*^{OPT} enables advanced genome engineering of both gene translation and transcription.

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COLLECTIONS

tetA dual selection protocols

KEYWORDS

Recombineering, counterselection, selection markers, genome engineering, synthetic biology, tetA

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PARENT PROTOCOLS

Part of collection

tetA dual selection protocols

Ordering of oligonucleotides

1 order the following oligonucleotides:

1.1 2 primers annealing in the *tetA* cassette including 50 bp overhangs that correspond to the regions up-and downstream of the locus that will be removed.

1.2 1 oligonucleotide (100bp) with the same 50 bp homology as the first primer set. This primer needs to anneal to the lagging strand. Use modest.biosustain.dtu.dk to create a sample "MAGE" oligonucleotide. Select your locus of interest in the dropdown menu "gene" and delete "A" in position 1. This will generate a MAGE oligonucleotide that will delete the A of the start codon in your gene that you want to delete. Align this oligonucleotide in your sequence software to see which strand it aligns to. This strand represents the lagging strand. Now create your custom oligonucleotide that aligns to the same strand as the MAGE sample oligonucleotide.

preculture and PCR - day 1 1d

2 Setup a preculture of the strain with pSIM19 (recombineering plasmid) in LB medium supplemented with Spectinomycin **0.05 mg/mL** and incubate at **250 rpm, 30°C** overnight. From now on the strain has to be kept at **30 °C** to maintain pSIM19 inside the cells.

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3 Prepare a PCR product of the *tetA* casette using a proof-reading polymerase and purify it.

Recombineering: tetA integration - day 2-4 3d

3d

4 Prepare:

4.1 Cold sterile water

4.2 Cold Glycerol [M]**15 % volume**

4.3 Pre-chilled centrifuge and tabletop centrifuge at **4 °C**

4.4 LB agar supplemented with [M]**0.05 mg/mL** tetracycline

4.5 M9 agar supplemented with [M]**50 micromolar (µM)** $NiCl_2$

5 Inoculate **50 mL** LB-Medium supplemented with Spectinomycin ([M]**0.05 mg/mL**) with **500 µL** of the preculture from step

5.1 Incubate at **250 rpm, 30°C** until cultures reached an OD600 of 0.5

6 Induce expression by transferring the culture to a shaking water bath at **150 rpm, 42°C, 00:20:00**

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7 Transfer culture to prechilled **50 mL** falcon tubes and put on ice for **00:15:00** 15m

8 Spin the culture down at **4000 x g, 4°C, 00:15:00** and discard the supernatant 15m

9 Add **1 mL** of ice cold water, resuspend and transfer to a **1.5 mL** tube

10 Spin at **11000 x g, 4°C, 00:00:30** in a tabletop centrifuge 30s

11 Wash pellet twice with **1 mL** ice cold water **go to step #9**

12 Resuspend the pellet in **600 µL** cold glycerol (**15 % volume**)

13 Unused cells can be stored at **-80 °C**

This is not possible for *E. coli* Nissle

14 Electroporate **50 µL** of cells with **200 ng** of purified PCR product from step 3

15 Recover cells **800 rpm, 30°C, 01:00:00** in a tabletop shaker using SOC medium.

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16 plate cells on LB agar supplemented with [M]**0.05 mg/mL** tetracycline. Cell might need up to 2 days to grow.

Recombineering: *tetA* removal - day 4 1d

17 Select a colony from the LB tetracycline plate and start a preculture in LB medium supplemented with Spectinomycin [M]**0.05 mg/mL** . Incubate at **250 rpm, 30°C** overnight.

18 prepare cells following steps 5-13

19 Electroporate **50 µL** of the prepared cells with **2 µL** of a [M]**100 micromolar (µM)** oligonucleotide

20 Recover cells at **800 rpm, 30°C, 01:00:00 in a tabletop shaker** . Afterwards, transfer the cells into **5 mL** LB medium supplemented with Spectinomycin

21 Incubate at **250 rpm, 30°C** overnight
Important! Cells need to lose tetA transporter in the membrane to get resistant to $NiCl_2$

Plating - day 5-7 2d

22 Wash **1 mL** of the recovered cells twice with sterile water. Centrifuge at **11000 rpm, 20°C, 00:00:30** 30s

23 Make a dilution series and plate **100 µL** of the 1:10 - 1:1000 dilution on M9 agar supplemented with [M]**50 micromolar (µM)** $NiCl_2$

24 incubate the plates at **30 °C** for **48:00:00** to **72:00:00** 5d

Cells can be incubated at **37 °C** if it is the last step of recombination and pSIM19 is no

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longer needed in the cells

Screening - day 8

25 Screen for positive colonies by colony PCR to identify the correct recombinants. Restreak correct colony on LB agar.

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