Oct 24, 2020

Testing safety mechanism

Andreea S[1]

[1]Unversity of Groningen

Other dx.doi.org/10.17504/protocols.io.bkuvkww6

iGEM Groningen 2020

a.stan.6

ABSTRACT

In order to test the efficiency of the kill switch mechanism, we plan on performing the following experiments:

Test amino acid autotrophy *in vitro*
An overnight culture will be used to inoculate a test culture. The knock out strains will be grown in a medium containing the test amino acid and then transfered to a minimal medium lacking the specific amino acid. Samples of 0.5 ml will be removed every 30 min to test the OD.
Testing solanine dependency *in vitro*
A test culture will be inoculated from the overnight culture. The cells will be grown in LB supplemented with solanine (CONCCCCCC). The cells will be collected, washed and resuspended in LB lacking solanine. Samples of 0.5 ml will be removed every 30 min in order to test OD.
Testing dependency in soil
The GFP-mutant strains (amino acid auxotrophs and solanine dependent mutants) will be inoculated at the root of the potato plants. The potato plants will be grown and described as above.

DOI

dx.doi.org/10.17504/protocols.io.bkuvkww6

PROTOCOL CITATION

Andreea S 2020. Testing safety mechanism. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bkuvkww6

CREATED

Sep 04, 2020

LAST MODIFIED

Oct 24, 2020

PROTOCOL INTEGER ID

41589

ABSTRACT

In order to test the efficiency of the kill switch mechanism, we plan on performing the following experiments:

Test amino acid autotrophy *in vitro*
An overnight culture will be used to inoculate a test culture. The knock out strains will be grown in a medium containing the test amino acid and then transfered to a minimal medium lacking the specific amino acid. Samples of 0.5 ml will be removed every 30 min to test the OD.
Testing solanine dependency *in vitro*

A test culture will be inoculated from the overnight culture. The cells will be grown in LB supplemented with solanine (CONCCCCCC). The cells will be collected, washed and resuspended in LB lacking solanine. Samples of 0.5 ml will be removed every 30 min in order to test OD.

Testing dependency in soil

The GFP-mutant strains (amino acid auxotrophs and solanine dependent mutants) will be inoculated at the root of the potato plants. The potato plants will be grown and described as above.

Test safety mechanisms in the lab

1 Plate mutants on minimal media supplemented with the auxotrophy amino acid or solanine.

2 Pick one colony and inoculate **5 mL** . Grow B. mycoides **Overnight** **200 rpm, 30°C**

3 The next day inoculate a working culture (**30 mL** media + 1% overnight culture)

4 Grow at **200 rpm, 30°C** until OD600nm is 0.5

5 Collect cells by centrifugation **4000 x g, 4°C, 00:10:00**

6 Wash 5 times with growth media (without amino acid)

This step may need ajustment. The purpose is to eliminate the dependency molecule from the growth medium

7 Resuspend in **30 mL** minimal media.

8 Divide culture in 2 x **15 mL** cultures.
Supplement one of them with the required amino acid.

9 Incubate both samples at **200 rpm, 30°C**

10 Every **00:30:00** take a **0.5 mL** sample and check OD600

Citation: Andreea S (10/24/2020). Testing safety mechanism. https://dx.doi.org/10.17504/protocols.io.bkuvkww6

11 The OD of the sample + amino acid will increase exponentially
The other one will remain 0.5

Test safety mechanism in soil

12 Grow potato plants in a 40 cm diameter pot.

13 Inoculate **5 mL** of a GFP-labeled B. mycoides (auxotroph) culture in exponential growing phase (OD600nm=0.5)
For a negative control use GFP-labeld B. mycoides.

Fluorescent labeling Bacillus mycoides
by r.rajadhyaksha

PREVIEW RUN

13.1 Routinely culture Bacillus strains in Luria-Bertani (LB) medium at **30 °C** with aeration at 200 rpm.

13.2

13.3 Prepare the B. mycoides strain aliquotes for electroporation.

13.3.1 Cultivate the bacterial strain overnight in LB broth at **30 °C** and at 180 rpm.

13.3.2 Transfer **1 mL** of the overnight culture into **100 mL** of LB medium (with 2% [wt/wt] glycine) and incubate it at **30 °C** and 180rpm until optical density at 600 nm is 0.4 to 0.7.

13.3.3 Centrifuge the cells and wash the pellets with increasing concentrations of ice-cold glycerol (2.5%, 5%, and 10%). Resuspend this pellet in precooled electroporation buffer (10% glycerol) and shock freeze in liquid nitrogen.

13.4 Add the library vector DNA in an amount of **2 µg** to the cells, and perform electroporation. The settings for electroporation are 2.0 kV, 25 lF and 200 X in a 2-mm cuvette using a Bio Rad Gen Pulser II electroporation system (Bio-Rad).

13.5 Add **1 mL** of LB medium and grow the cells for **02:00:00** at **30 °C** and 150 rpm for recovery and then plate on LB-Cm4 agar.

Citation: Andreea S (10/24/2020). Testing safety mechanism. https://dx.doi.org/10.17504/protocols.io.bkuvkww6

13.6 After **24:00:00** of growth at **30 °C** , harvest the colonies from the plates and pool in LB medium

13.7 Store the libraries at **-80 °C** as 15% glycerol stocks.

13.8 Inoculate the B. mycoides strain *mKate2mut* library in **50 mL** of LB-Cm4 and grow at **7** or **6** to an OD_{600}nm of 0.3-0.6.

13.9 B. mycoides has been seen to show extensive cell-chaining and hence a mild sonication step of 4 rounds of 3 X 10 pulses of 1s with an amplitude of 30% can be applied to disassemble the aggregated cells.

13.10 Sort the cells on a flow cytometer at 20 psi using a **70 Micromolar (µM)** nozzle at a flow rate of 1.0 with the highest sort precision mode (0– 32-0 sort purity mask).

13.11 Using a sequential gating strategy with FCS height versus widths, followed by SCC height versus width, cellular debris, and chained cells can be excluded.

13.12 To separate the brightest variants choose a cutoff of 3% of the brightest event in the first round of cell sorting and 0.3% of the brightest events in the second round of sorting with the light scatter parameters.

13.13 After FACS sorting, plate the final fluids containing bright cells on LB-Cm4 plates and grown them overnight at **30 °C** .

13.14 Observe the colonies using a fluorescence microscope. Keep the filter setting for GFP as excitation at 460/480nm and emission at 495/540 nm with a 485 nm dichromatic mirror; for RFP, the filter setting can be kept as excitation at 545/580 nm and emission at 610 nm with a 600 nm dichromatic mirror.

13.15 Capture the images on a camera and calculate the intensity of single-cell with Image J software.

13.16 Calculate the total cell fluorescence the formula is: Corrected total cell fluorescence (CTCF) = Integrated Density – (Area of selected cell x Mean fluorescence of background readings)

14 Take soil samples of control and mutants from:
- as close to the root as possible
- 10 cm distance fro m root
- 20 cm distance from root
- 30 cm distance from root
- 40 cm distance from root

Citation: Andreea S (10/24/2020). Testing safety mechanism. https://dx.doi.org/10.17504/protocols.io.bkuvkww6

- 50 cm distance from root

Citation: Andreea S (10/24/2020). Testing safety mechanism. https://dx.doi.org/10.17504/protocols.io.bkuvkww6