Protocol S3:

A new range of vectors with a stringent 5-fluoro orotic acid-based counterselection system for generating mutants by allelic replacement in *Staphylococcus aureus*.

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Detailed protocol for temporary disruption of the *pyrE* gene, using plasmid pRLBER9:

Summary:

pRLBER9, can be transformed into any *S. aureus* strain (via RN4220 if DNA modification is needed), whereupon a shift to 42°C in erythromycin will force the plasmid into the *pyrFE* genes, thus disrupting the *pyrE* gene. This event can also be selected for with FOA, which will additionally prevent the plasmid from recombining back out of the chromosome. The resulting *pyrE*$^-$ strain can be used for allelic replacement using the pRLY-vectors, since there is no sequence similarity between them and the pRLB-vectors. Once the desired mutation has been obtained using the pRLY-vectors, then pRLBER9 can be removed from the *pyrFE* genes by selecting on RH-medium, and then the plasmid can be lost by shifting the temperature to 42°C. This is a very frequent event (figure S2), since pRLBER9 does not confer any advantage to the cell, and plasmid-loss can easily be screened for by erythromycin sensitivity.

Mediums used:

MH: Mueller-Hinton

MHC: MH with 10 µg/ml chloramphenicol

MHE: MH with 10 µg/ml erythromycin

MHFOA: MH with 200 µg/ml 5-fluoro-orotate

MHEFOA: MHFOA with 10 µg/ml erythromycin

MHU: MH with 10 µg/ml uracil

MHEU: MHE with 10 µg/ml uracil

RH: Sigma R7388 (basically RPMI-1640 medium with 20 mM Hepes and L-glutamine)

Agar is used at 13 g/l

This protocol is an example, using pRLBER9.

Modify the plasmid DNA before transforming into a wild type *S. aureus*:

Many *S. aureus* strains encode restriction system, and to ensure high transformation frequencies it is important to modify the plasmid DNA before transforming into a wild type *S. aureus* strain. This is classically done by passing the plasmid through the restriction deficient *S. aureus* strain RN4220.

Once the pRLBER9-DNA is correctly modified, it is transformed into the desired *S. aureus* strain, remembering that the plasmid has a thermosensitive origin of replication which works best at 30°C.

Introducing pRLBER9 into the *pyrFE* locus.

1) pRLBER9 is transformed into the desired strain for genetic manipulations, and selected on MHE. A transformant colony is then transferred to 3 ml MHE, and incubated over night at 30°C.

2) The next day, 10 µl of the culture is added to 5 ml fresh MHE, which is incubated for 6 hours at 42°C.

3) 50 µl is plated on half a MHE-plate, and an inoculation-loop is used to “streak” for single colonies on the remaining half. The plate is incubated over night at 42°C.

4) A few single colonies are picked, and restreaked on a MHE-plate at 42°C

5) Restreaked colonies are resuspended in 500 µl MHE in a 2 ml eppendorf tube. 5 ul spots are deposited on MH, MHE, MHFOA and RH (it is possible to do an extra check by spotting on RH+Erythromycin). Plates and eppendorfs are incubated at 42°C over night (the tubes should be agitated).

6) Strains that have pRLBER9 correctly recombined into *pyrFE* will grow on MH, MHE and FOA. Growth will normally also be seen on RH-plates, since RH will select for plasmid cross-out, however, RH+Erythromycin should not allow growth.

7) 20 µl from an eppendorf which grew correctly on the plates, is streaked on MHFOA at 37°C over night.

8) A single colony is picked, and transferred to 5 ml MHFOA. Incubate with agitation at 37°C over night.

9) Make -80°C stock of the strain, and use some to start a culture for making competent cells. Keep FOA selection up, to keep the pRLBER9-plasmid integrated into the chromosome.

The competent cells can now be used for allelic replacement with the pRLY-vectors, using Protocol S1.

Once all desired allelic replacements have been carried out, then it is time to eliminate the pRLBER9-plasmid, and reconstitute the *pyrFE* locus.

Eliminating pRLBER9, after the completion of allelic replacements:

10) 30 µl of an over night culture is added to 5 ml RH-medium, and incubated with agitation for 6 hours at 42°C.

11) 50 µl of the culture is plated on half a RH-plate, and an inoculation-loop is used to streak for single colonies on the other half. The plate is incubated at 42°C over night.

12) A few colonies are picked, and restreaked on RH at 42°C over night.

13) Loss of pRLBER9 is checked by spotting on MH, MHE, MHFOA and RH. Growth on MH and RH, but not on MHE and MHFOA signifies loss of the plasmid.