Protocol S2:

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 using pRLSAYx9 vectors:

pRLSAYT9 is used as example.

Mediums used:
MH: Mueller-Hinton
MHT: MH with 2 µg/ml tetracyclin
MHFOA: MH with 200 µg/ml 5-fluoro-orotate
MHT+FOA: MHFOA with 10 µg/ml erythromycin
MHU: MH 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

1) pRLSAYT9 is transformed into the *pyrFE*$^{-}$ strain, and colonies are selected on MHT.

2) The transformants are restreaked once on MHT to ensure clonal purity.

3) 10 µl of an overnight liquid culture (in MHT) is added to 5 ml MHT+FOA and incubated with agitation for 6 hours at 42°C (the FOA is to ensure that pRLSAYT9 recombines into the region of homology which is downstream of the *pyrFE* mutation, and therefore does not complement the *pyrFE*$^{-}$ phenotype).

4) 50 µl of the culture is plated on MHT+FOA at 42°C.

5) Colonies are restreaked on MHT+FOA at 42°C. These colonies can be checked by spotting on MH, RH, MHT and MHFOA, there should be no growth on RH.

6) 10 µl of an overnight liquid (MHT+FOA) culture is added to 5 ml MH medium and incubated with agitation for 6 hours at 42°C.

7) 50 µl of the culture is plated on RH, at 42°C.

8) Colonies are restreaked on RH at 42°C.

9) Restreaked colonies are resuspended in 500 µl RH, and 5 µl spots are spotted on MH, RH, MHT and MHFOA-plates at 30°C.

10) Strains that grow on MH and RH, but NOT on MHT or MHFOA are restreaked once more, to ensure clonal purity.

11) PCR the *pyrFE* region and sequence it, to verify the reversion to wild-type.