Protocol S1:

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 the pRLY-vectors:
The example given is using pRLYE1 for replacements in strain PR01 (*S. aureus* SA564 Δ*pyrFE* and restriction enzymes disrupted).
For replacing an allele with an antibiotic resistance marker, see next example.

General information:

Construct: About 1-1.5 kb on each side of the target-region, is cloned into the MCS (from *Nde*I to *Xho*I) of pRLYE1, in the same relative orientation as they appear on the chromosome.

Note I: We generally clone genes anti-clockwise in the pRLYE1-sequence, following the direction of all the major ORFs in the vector.

Note II: Blue-white screening in *E. coli* is possible with the pRLY-vectors.

Mediums used:
MH: Mueller-Hinton
MHC: MH with 10 µg/ml chloramphinicol
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

Bead-beating: 250 µl TE with 10-20 small glass beads is placed in an eppendorf tube. A colony is picked and resuspended in the TE, whereupon the tube is vortexed for at least 1 minute. 1-2 µl of the suspension can be used for PCR-template.

Protocol I:

Transformation into *S. aureus*:

1) 1-2 µg of plasmid is dried in a speedvac in a 2ml eppendorf tube.

2) Then 70 µl competent cells are added to the tube, which is "flicked" to dissolve the DNA, and then left for 4 mins at room temperature.

3) The cells with the DNA are transferred to a 2 mm electroporation cuvette, and electroporated at 2.3 kV, timeconstant 2.5 ms.

4) Immediately add 1 ml MH-medium (Mueller-Hinton-medium), and shake at 37°C for 90 mins.

5) Spread 4x200 µl of the transformation culture on MHE-agar-plates. Incubate at 37°C for 24-48 hours

> Note III: We usually see a few colonies after 24 hours, and about twice that number after 48. If the pyrFE mutant used has a low reversion-rate, then RH-plates can sometimes be used with advantage to select for transformants. Additionally, it is also possible to select in liquid culture (MHE or RH), which sometimes helps to obtain transformants.

6) Pick 10-20 colonies (or as many as there are), and restreak them on MHE-plates

> Note IV: If the colonies do not grow well when restreaked, then restreak one more time, and if a colony does not grow "normally", then consider dropping it at this stage).

7) Resuspend the restreaked colonies in 500 µl MHE each in a 2 ml eppendorf tube, and vortex for 10 seconds.

8) Use the 500 µl tubes to:

8a) Spot 5 µl of the culture on MH, MHE, MHFOA and RH-plates

> Note V: make sure plates are nice and dry before spotting)

8c) Incubate the remaining culture 37°C over night with agitation, while keeping the tubes horizontal to maximize aeration.

9) Strains that are “positive”, will grow on MH, MHE and RH, but NOT on MHFOA. Discard strains that are not “positive”.

10) Pass 10 µl of the “positive” cultures to 3 ml fresh MHU (done in the morning). Shake at 37°C. Use what remains of the 500 µl cultures to make -80°C stocks.

11) Use a bit of cells from the MHE spots to bead-beat in TE, and then do an EXT-INT-L PCR and an INT-EXT-R PCR (see figure S4). If you have some strains with insert in L and others with insert in R, then continue with one of each in step 12, otherwise continue with two of the same

> Note VII: Sometimes either L or R-PCR, or both, do not give a product. Some regions are hard to design good primers for or are hard to amplify, you can continue “blind” or do a total DNA preparation to use as template.

12) In the evening, use 50 µl of the 3 ml MHU-culture from step 10 to plate on half a plate of MHFOA, use a loop to streak the culture so you will have “diluted” on the same plate. Incubate at 37°C over night.

13) Restreak colonies on MHFOA-plates

> Note VIII: Colonies may appear late in the day.
>
> Note IX: It is usually practical to pick 10-15 colonies from each strain.

Make sure to take both small and large colonies, the wanted mutation might inhibit growth. Incubate at 37°C over night.

14) Resuspend a single colony from each restreak into 500 µl MHFOA in a 2ml eppendorf tube, then spot 5 µl on MH, MHE, MHFOA and RH-plates. Incubate at 37°C over night. Put the eppendorf-tube with the remaining resuspension at 37°C shaking over night (keeping the tubes horizontal).

15) Strains that grow on MH and MHFOA, but NOT on MHE and RH are possible positives for step 16

> Note X: If you are not using a pyrFE deletion, but rather a point mutation, then a

small amount of revertants might be seen in the RH-spots.

If there is a known visual phenotype of the mutation (e.g. no growth without tryptophan), then spot on an additional plate to detect that.

16) Use the spot from MHFOA to:

16a) (optional I) Use a small amount to bead-beat, and do an EXT-INT-L and an INT-EXT-R-PCR If you have a product in one or both, then you are very likely to have your mutation

Note XI: Either L or R-PCR might not work, so “no product” does not mean “no deletion”, 16b is the “real” test.

16a) (optional II) Alternatively, do an INT-INT-PCR, this PCR is shorter, and therefore less sensitive to template quality. Additionally, most INT-INT primer pairs will yield a longer product from the revertants to wild-type (marker-loss due to homologues recombination via the same region as the integration).

16b) Prepare total DNA of positives from step 16a (or from all), using the Blood and Tissue Kit (Qiagen)

17) Use the total DNA from step 16b to do an EXT-EXT PCR, use wild-type DNA as control.

18) Restreak 10 µl of the overnight cultures in the 2 ml eppendorf tubes (from step 14) of the positive clones (from step 16a) on MHFOA.

19) Sequence PCR product from step 17, to ensure that a) the correct sequence has been amplified, and b) there are no secondary point mutations in the region.

20) Further restreak the positive clone(s) (from step 18) at least 3 times on MHFOA, and make -80°C stock.

Protocol II:

(When using a resistance-cassette for exchange)

In this example pRLYC1 will be the basis vector, and an erythromycin cassette will be exchanged for the deleted gene.

Steps 1-4 are the same as in the previous example.

5) Spread 2x200 µl of the transformation culture on MHE-agar-plates and 2x200 µl on MHC-plates. Incubate at 37°C for 24-48 hours

Note III: We usually see a few colonies after 24 hours, and about twice that number after 48. If the pyrFE mutant used has a low reversion-rate, then RH-plates can sometimes be used with advantage to select for transformants. Additionally, it is also possible to select in liquid culture (MHE or RH), which sometimes helps to obtain transformants.

6) Pick 10-20 colonies (or as many as there are), and restreak them on MHE-plates if they are picked from an MHC-plate, and on MHC if they are picked on an MHE-plate

Note IV: If the colonies do not grow well when restreaked, then restreak one more time, and if a colony does not grow “normally”, then consider dropping it at this stage.

7) Resuspend the restreaked colonies in 500 µl MHE each in a 2 ml eppendorf tube, and vortex for 10 seconds.

8) Use the 500 µl tubes to:

8a) Spot 5 µl of the culture on MH, MHE, MHC, MHFOA, MHEFOA and RH-plates

Note V: Make sure plates are nice and dry before spotting

8c) Incubate the remaining culture 37°C over night with agitation, while keeping the tubes horizontal to maximize aeration.

9) Strains that are “positive”, will grow on MH, MHE, MHC and RH, but NOT on MHFOA and MHEFOA. Discard strains that are not “positive”.

10) Pass 10 µl of the “positive” cultures to 3 ml fresh MHEU (done in the morning). Shake at 37°C. Use what remains of the 500 µl cultures to make -80°C stocks.

11) Use a bit of cells from the MHE spots to bead-beat in TE, and then do an EXT-ERY-L PCR and an ERY-EXT-R PCR (see figure S4). If you have some strains with insert in L and others with insert in R, then continue with one of each in step 12, otherwise continue with two of the same

Note VII: Sometimes either L or R-PCR, or both, do not give a product. Some

regions are hard to design good primers for or are hard to amplify, you can continue “blind” or do a total DNA preparation to use as template.

12) In the evening, use 50 µl of the 3 ml MHEU-culture from step 10 to plate on half a plate of MHEFOA, use a loop to streak the culture so you will have “diluted” on the same plate. Incubate at 37°C over night.

13) Restreak colonies on MHEFOA-plates (Note VIII: Colonies may appear late in the day.

Note IX: It is usually practical to pick 10-15 colonies from each strain. Make sure to take both small and large colonies, the wanted mutation might inhibit growth. Incubate at 37°C over night.

14) Resuspend a single colony from each restreak into 500 µl MHEFOA in a 2ml eppendorf-tube, then spot 5 µl on MH, MHC, MHE, MHFOA, MHEFOA and RH-plates. Incubate the plates at 37°C over night. Put the eppendorf-tube with the remaining suspension at 37°C with shaking over night (keeping the tubes horizontal).

15) Strains that grow on MH, MHE, MHFOA and MHEFOA, but NOT on MHC and RH are possible positives for step 16

Note X: If you are not using a *pyrFE* deletion, but rather a point mutation, then a small amount of revertants might be seen in the RH-spots.

16) Use the spot from MHEFOA to:

16a) Use a small amount to bead-beat, and do an EXT-ERY-L and an ERY-EXT-R-PCR If you have a product in one or both, then you are very likely to have your mutation

Note XI: Either L or R-PCR might not work, so “no product” does not mean “no deletion”, 16b is the “real” test.

16b) Prepare total DNA of positives from step 16a (or from all), using the Blood and Tissue Kit (Qiagen). This can also be done by using the restreak from step 18.

17) Use the total DNA from step 16b to do an EXT-EXT PCR, use wild-type DNA as control.

18) Restreak 10 µl of the overnight cultures in the 2 ml eppendorf tubes (from step 14) of the positive clones (from step 16a) on MHEFOA

19) Sequence PCR product from step 17, to ensure that a) the correct sequence has been amplified, and b) there are no secondary point mutations in the region.

20) Further restreak the positive clone(s) (from step 18) at least 3 times on MHEFOA, and make -80°C stock.

Notes on designing the homologous regions for cloning into the pRLY-vectors:

The choice of homologous L and R regions for the deletions/substitutions should be done by considering length of the regions as well as suitable priming sites for the PCR amplification of the regions. To avoid strong bias towards recombining via one over the other, the L and R should generally be of approximately the same length. Nevertheless, we have observed that even with L and R regions of the same length, there is often a bias in which region recombines more readily.

An example of a special case was the *cshA*-substitution (figure 6 in the manuscript), where an adjacent IS element prevented a long R-region. The L-region was therefore elongated to ~1800 bp, to ensure that the first recombination would be via L, and the Cm-marker was then used to force the second recombination to take place via the much shorter R (~600 bp).