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Triparental mating with pSEVA protocol V.2

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We use this protocol and it's working

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Abstract

This process involves bacterial conjugation, where a conjugative plasmid found in one bacterial strain facilitates the transfer of a mobilizable plasmid from a second bacterial strain to a third bacterial strain.
In the method from our lab, CC118λpir containing a pSEVA plasmid works as the donor, the *E. coli* 1047 pRK2013 strand is the helper, and the receiver is a *C. rodentium* strand.

Insertion of he suicide plasmid by three partner conjugation

3d 2h 55m

1 Inoculate Overnight cultures of strains: 1d

DONOR	CC118λpir pSEVA	Gm (10 µg/ml)
HELPER	*E. coli* 1047 pRK2013	Km (50 µg/ml)
RECEIVER	*C. rodentium* pACBSR	Sm (50 µg/ml)

Note

We have a pACBSR plasmid encoding Cm resistance.

2 Place 20 µl spots of the helper and donor strains onto an LB plate (no antibiotics) and an additional spot of **20 µl of the helper on top of 20 µl of the donor (D+H)**. 10m

3 Leave the plate open at the flame until the spots get dry. Incubate at 37 °C 02:00:00, facing up. 2h

4 Add **40 µl of the receiver strain on top of the D+H spot (D+H+R)** and an additional 20 µl spot of this strain alone. Wait for the spot to get dry and incubate for 04:00:00 37 °C facing up. 4h

5 Collect the 4 patches using a sterile loop and resuspend each of them in 1 ml of LB in an eppendorf. 10m

6 Plate 100 µl of each tube in LB plates supplemented with **Gm + Sm**. 10m

7 Centrifuge the rest of the D+H+R tube at 2000 x g, 00:02:00 to pellet the cells, resuspend in 100 µl and plate as well in LB plates supplemented with **Gm + Sm**. 5m

8 Incubate the plates Overnight at 37°C. 1d

Note

Due tto the inability of pSEVA vector to replicate in *C. rodentium*, the only way to grow on Gm plates is having the pSEVA vec tor integrated into the chromosome (cointegrate).

Second recombination for a scarless genomic modification

2d 10h 10m

9 Pick two colonies of the D+H+R plate and grow them in **LB+Sm + 0.4% L-arabinose broth** for a minimum of 06:00:00 for the induction of the I-SceI endonuclease of the pACBSR plasmid. 6h

Note

In the Overnight plates with only donor, helper or receiver no colonies should grow.

10 Insert the inoculation loop in the culture and streak on LB+Sm plates to obtain individual colonies. Incubate the plates Overnight at 37°C. 1d 0h 10m

11 The next day, pick some colonies and patch them on a **LB+Sm** plate and on a **LB+Sm+Gm** plate. Incubate the plates Overnight at 37°C. 1d 0h 30m

Note

Colonies that do not grow on the plate with Gm are those that have recombined after treatment with the endonuclease I-SceI.

12 Analyze by PCR and gel electrophoresis those colonies which have grown on LB+Sm but not on LB+Sm+Gm to differentiate the modified colonies from the ones which have reverted to the Wild-Type genotype. 3h 30m

Note

The primers should hybridise outside of the homology regions selected. Upon analysis of 10 colonies you should get a about 50% of modified colonies.

13 To remove the pACBSR plasmid, make 8-9 passes of the strain without Sm in liquid LB. Plate the last culture in LB plates and patch individual colonies the following day on LB and LB+Sm plates to select those sensitive to the antibiotic.