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We use this protocol and it's working

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Triparental mating with pSEVA protocol

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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.

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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 c 10m
20 µl of the helper on top of 20 µl of the donor (D+H).

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 s 1d 0h 20m
alone. Wait for the spot to get dry and incubate for Overnight 37 °C facing up.

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. 5m

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

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 + L-arabinose broth at 0.4%** 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. In 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. Incu 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 3h 30m to differentiate the modified colonies from the ones which have reverted to the Wild-Type genotype.
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 **NOTE**: 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.