Tri-parental Matings
For introduction of broad host-range plasmids into bacteria via conjugation from *E. coli*

Some typical conditions:
"Donor" = *E. coli* strain carrying broad host-range cloning vector. (pKT230)
"Helper" = *E. coli* strain carrying RP4
"Recipient" = *Rhizobium;* incubated at 30C on Tryptic Soy Broth (TSB)
Selection will often involve two antibiotics. One marker is carried on broad host-range plasmid, such that all helper and recipient strains that have not received this plasmid can be killed. The other marker is carried on the chromosome of the recipient and allows counterselection against donor and helper regardless of whether or not they carry the broad host-range plasmd.

- pKT230: kan , strep resistance
- selection media: m9 sucrose + kan + strep

Start Cultures:
1. Grow fresh overnight cultures of donor, recipient and helper bacterial strains. Use selectable markers (i.e., add appropriate antibiotic to media). Liquid cultures are optimal but cells can also be grown on plates.
    a. For RP4, cultures must be grown on the day of

Harvest and Wash Cells:
2. About 10^9 *Sinorhizobium* and 0.5×10^9 *E. coli* cells were used for each mating.
Remove 1 ml of liquid culture to a 1.5 ml microcentrifuge tube. Pellet cells (donor and recipient separate) by spinning 5 min. at 8000 rpm. Decant liquid and save pellet.

A cloudy supernatant is OK - you do not need all of the cells.

3. Wash cells twice with Tryptic Soy
4. Mix both donor and recipient cells, pellet again and resuspend in 0.1 mL of TSB
5. Normalize the cell density in culture for each strain by diluting 10 µl of culture in 990 µl of fresh medium in a spectrophotometer cuvette. Check the OD at 600 nm on the spectrophotometer and calculate the amount of medium to add with the following equation: medium to add in µl = $(OD_0 \times V_0)/(OD_F – V_0$, where OD_0 is the current OD_{600} value, V_0 is the current volume that the cells are suspended in and OD_F is the desired OD_{600} value.
6. Transfer mating cells to TSB plate (two 20uL spots, six 6 uL spots) and incubate at 30C for 4h
7. Recover cells from plate with a sterile handle and resuspend in 1mL of TSB

Combine Strains for Mating:
5. Plate on selective and nonselective media. Mark one or more petri plates with areas that will hold each donor+helper+recipient combination, as well as controls that will hold each strain individually and combinations such as donor+helper and helper+recipient.

Generally it is good to use plates that are dry enough to absorb liquid but still fairly moist and fresh. The media should not be selective at this time. Six to twelve conjugations fit nicely onto a single 15 x 100 mm plate.

6. Spot washed cells onto plates at appropriate positions. 5 ml per strain is often convenient. For strain combinations, no mixing is necessary - just add cells together in one puddle.

   Be careful not to splash droplets as you add cells. After the first strain has been added, be sure to change pipet tips between each spot. A pre-mix can be made, for instance containing helper+recipient.

7. If plate is too moist, allow time for absorption or dry briefly in sterile hood. Do not bump plate or allow separate spots to run together.

8. Incubate plates face-up for 3 days at 30^{O}C.

Select Desired Exconjugants:

9. Using a toothpick or loop, remove some cells from conjugation spot and streak for single colonies on selective media (auxotropic or antibiotic - see above). Be sure to also streak out controls.

10. Incubate plates for two days at 30^{O}C. Pick single colonies and re-streak on selective media to purify.

Confirm exconjugants by DNA analysis, and don't forget to make frozen permanent cultures of the strains.

Efficiency of conjugation was estimated as the ratio between the number of transconjugants and the number of donor cells.