CONJUGATION:

1. Several colonies of the recipient strain (e.g., *M. trichosporium* OB3b) are inoculated into 50 mL of NMS medium (containing antibiotics for any marker-exchange mutant used as recipient) in a 250-mL side-arm flask under methane. The culture is incubated at 30 C with shaking (225 rpm). NOTE: temperature can be changed for recipient, e.g., 45C for *M. capsulatus* Bath.

2. The cells are harvested when OD540 is 0.2-0.3 (generally takes between 24 h and 96 hours). The methanotrophs are harvested by centrifugation (7000g, 5 min, room temperature).

3. The donor strain (S17-1) is grown overnight from a single colony in LB medium (plus antibiotics to select for the plasmid), centrifuged as above for collection.

4. Both recipient and donor cells are washed with 50 mL of fresh NMS medium without antibiotics, and resuspended in 10 mL of the same medium.

5. The suspensions of washed donor and recipient cells are mixed and the cells are collected onto a 47-mm diameter sterile nitrocellulose filter (0.2 mm pore size), by using a sterile filter unit connected to a vacuum pump.

6. A pair of stainless steel tweezers sterilized by dipping in absolute ethanol and flaming to remove the ethanol are then used to transfer the filter, **bacteria-side-up** onto a plate of NMS agar plus **0.02% (w/v) Proteose Peptone**.

7. The plate with filter is incubated for 24 h-**48 h** at 30 C in an air/methane atmosphere. NOTE: for mild thermophilic methanotrophs this should be increased to 37C (or higher).

8. Using a pair of sterile tweezers and a sterile Falcon tube, the bacteria are then washed from the filter into 10 ml of fresh sterile NMS medium.

9. The suspension is concentrated by centrifugation (7000*g, 5 min, room temperature) and the cells are resuspended in 1mL of NMS.

10. Plate and spread 200uL aliquots on NMS plates containing proper antibiotics to select for transformants. Also include a negative control (i.e. wild type methantroph) with same amount of suspension.

11. Incubate the plates at 30C with methane/air. The colonies may appear after 2-3 weeks. Conjugation frequencies vary greatly, typically from 1 to 100 colonies per plate.

12. Colonies are picked and streaked onto fresh NMS plates containing antibiotics and 10ug/mL nalidixic acid. Incubate at 30C. Purified exconjugants are usually visible within 2 weeks.

We have found that this two-stage selection procedure is necessary. Our attempts to shorten the selection procedure by plating the exconjugants initially on plates containing more than one antibiotic have not yielded exconjugants.

Reference: Mutagenesis of Soluble Methane Monooxygenase; Thomas J. Smith and J. Colin Murrell, Chapter 9. Methods in Enzymology; Volume 495, 2011, Pages 135–147; Methods in Methane Metabolism, Part B: Methanotrophy.

Alternatively, instead of using the filtration method, spotting method can be utilized for steps 5 – 10 as below.

5. Suspensions of washed donor and recipient cells were mixed.

6. The mixed suspension is spotted (50 µL/spot) on NMS agar plus 0.02% (w/v) Proteose Peptone.

7. The plate is incubated for 24 h-48 h at 30 C (or higher as needed) in an air/methane atmosphere.

8 - 9. Spots are scraped off the agar with sterile sticks and resuspended in 1 mL of NMS.

10. Spread 200 µL aliquots on NMS plates containing proper antibiotics to select for transformants.