**DNA Digestion:**

1. DNA sample is mixed with 2.5 µl EcoRI buffer (10x) and water is added for a final volume of 23 µl; 2 µl EcoRI restriction enzyme added.
2. Incubate sample overnight at 37˚C in PCR machine (Protocol: 37˚F for ever).
3. Mix sample with 8 µl gel loading buffer and load on 0.7% agarose gel with 4 µl size marker. Run at 120 V for 20 minutes.
5. Visualize DNA on the transilluminator ( CAREFUL - UV LIGHT !, WEAR PROPER FACE SHIELD AND LAB COAT FOR SKIN PROTECTION ) and cut out the agarose fragments from 1500 – 5000 bp. DNA size marker in the flanking lanes will Indicate the right size range.
6. Use QIAquick Gel extraction kit (QIAGEN) to recover DNA fragments from gel matrix. Elute the DNA with 50 µl EB buffer.
7. Precipitate DNA: Mix sample with 5 µl Na-acetate (pH 5.2, 3M), 125 µl EtOH, and 2 µl pellet paint (Novagen).
8. Store at –20˚C for 20 minutes.
9. Centrifuge at 14,000 rpm at 4˚C for 15-20 minutes.
10. Remove supernatant and wash pink pellet with 1 ml EtOH 70%.
11. Centrifuge at 14,000 rpm at 4˚C for 15-20 minutes.
12. Remove supernatant and let pellet air-dry for 15-20 min.
13. Rehydrate DNA with 10 µl EB buffer.

**Cloning vector preparation: (FYI only)**

The pBluescript-SK+ vector from Stratagene, is used as shuttle system for our metagenomic fragments. The plasmid was linearized with EcoRI and the ends were dephosphorylated to reduce self-religation.

1. Mix 5 µg vector (~6 µl) with 5 µl of EcoRI buffer and adjust volume to a total of 46 µl with water.
2. Add 4 µl EcoRI and incubate for overnight at 37˚C.
3. Dilute reaction mixture with 50 µl water and add 2 µl Shrimp alkaline phosphatase. Continue incubation for 2 hours.
4. Mix sample with 20µl Gel loading buffer and load entire sample on 0.7% agarose gel with the two wide wells. Divide the sample between the gels. Load 4µl of Lambda marker in each of the small wells alongside.
5. Visualize DNA on the transilluminator and recover DNA fragment around 3,000bp.
6. Use QIAquick Gel extraction kit to recover DNA fragment from gel matrix. Elute the DNA with 50 µl EB buffer.
7. Precipitate Vector with EtOH.

**Ligation:**

1. eDNA sample (10µl) is mixed with 1 µl linearized pBluescript-SK+ (from above), 3 µl T4 ligase buffer (10x), 13 µl water, and 3 µl T4 DNA ligase.
2. For a control, mix 1 µl linearized pBluescript-SK, 3 µl T4 ligase buffer (10x), 23 µl water, and 3 µl T4 DNA ligase.
3. Incubate samples overnight at room temperature.
4. Store at 4˚C