**Genomic DNA Purification & Restriction Digestion:**

Prior to digestion of the eDNA sample, the material is cleaned with the Wizard® Genomic DNA kit from Promega.

1. Genomic DNA sample (20μl) is mixed with 600 μl Lysis solution – mix well
2. Incubate sample for 5 minutes at 80˚C
3. Cool to room temperature
4. Add 3 μl of RNase A (Promega) and incubate the mixture for 60 minutes at 37˚C
5. Cool to room temperature
6. Add 200 μl Protein precipitation solution – mix very well
7. Centrifuge at room temperature for 3 minutes (13,400 rpm)
8. Transfer clear supernatant to tube containing 600 μl isopropanol – mix well and let sample sit at room temperature for 15 minutes.
9. Centrifuge at room temperature for 3 minutes (13,400 rpm), then discard supernatant
10. Wash pellet with 600 μl Ethanol 70%
11. Centrifuge at room temperature for 3 minutes (13,400 rpm), then discard supernatant
12. Let pellet air-dry for 15 minutes, then add 10 μl Tris-buffer and store at 4˚C

**eDNA Digestion:**

13. DNA sample (10μl) is mixed with 2.5 μl BamHI buffer (10x), 10.5 μl water, and 2 μl BamHI restriction enzyme.
14. Incubate sample overnight at 37˚C
15. Mix sample with 8 μl gel loading buffer and load on 0.7% agarose gel.
16. Run at 120 V for 20 minutes
17. 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 help chose the right size range.
18. Use QIAquick Gel extraction kit to recover DNA fragments from gel matrix. Elute the DNA with 50 μl EB buffer.
19. Mix sample with 5 μl Na-acetate (pH 5.2, 3M), 125 μl EtOH, and 2 μl pellet paint (Novagen).
20. Store at –20˚C for 20 minutes
21. Centrifuge at 14,000 rpm at 4˚C for 15-20 minutes.
22. Remove supernatant and wash pink pellet with 1 ml EtOH 70%
23. Centrifuge at 14,000 rpm at 4˚C for 15-20 minutes.
24. Remove supernatant and let pellet air-dry for 15-20 minutes
25. Rehydrate DNA with 10 μl EB buffer.

**Cloning vector preparation:**

The pBluescript-SK+ vector from Stratagene (discontinued) is used as shuttle system for our metagenomic fragments. The plasmid was linearized with BamHI and the ends dephosphorylated to reduce self-religation. BamHI was chosen based on the literature as the enzyme seems least sensitive to inhibitors.

1. Mix 5 μg vector (~6 μl) with 5 μl of BamHI buffer and adjust volume to a total of 46 μl with water.
2. Add 4 μl BamHI and incubate for 5-6 hours at 37˚C.
3. Dilute reaction mixture with 50 μl water and add 2 μl Shrimp alkaline phosphatase. Continue incubation for 2 hours.
4. Purify DNA sample with QIAquick PCR purification kit and elute sample with 50 μl EB buffer.
5. Final concentration: ~80 ng/μl; stored at 4˚C.

**Ligation:**

26. eDNA sample (10μl) is mixed with 1 μl linearized pBluescript-SK+ (see above), 3 μl T4 ligase buffer (10x), 13 μl water, and 3 μl T4 DNA ligase.
27. Incubate sample overnight at room temperature.
28. Store at 4˚C