Bacterial community fingerprinting by T-RFLP analysis:

After extraction and purification of total DNA from soil, the bacterial 16S small subunit rRNA gene is amplified using primer set F27 (5′ -AGAGTTTGATCMTGGCTCAG - 3′) and R1492 (5′ - TACGGYTACCTTGTTACGACT - 3′) (Lane et al. 1985). The forward primer F27 is labelled with 6-FAM.

PCR:

PCR reactions are performed in 50 μl volumes containing

Component	**Volume (μL) for 1 sample**
10X Takara Ex Taq PCR buffer w/MgCL2	5
Forward primer (3μM)	5
Reverse primer (3μM)	5
BSA (5 mg/ml)	5
dNTP mix Takara (2.5mM)	4
Takara ExTaq DNA polymerase (5 U/μL)	0.2
ddH_2O	25.3
DNA template	0.5
	50

1. The thermocycling conditions are as follows: an initial melt at 95°C for 3 min (1 cycle); 95°C for 30 s, 53°C for 30 s, 72°C for 1 min (up to 35 cycles); with a final extension at 72°C for 7 min and infinite hold at 4°C.
2. PCR products (5μl) are visualised on a 1 % agarose gel and then purified using a MoBio PCR product purification kit and eluted in a final volume of 45μL.

Restriction digestion:

The purified PCR product (and negative control) is then digested enzymatically overnight in the 37oC incubator in 0.5 ml tubes:

Component	AluI digest (µl)	MspI digest (µl)	HhaI digest (µl)
PCR product*	~ 400 ng	~ 400 ng	~ 400 ng
10 x buffer	5 (NEB buffer #2)	5 (NEB buffer #2)	5 (NEB buffer #4)
BSA (10 mg/ml)	-	-	0.5 µl
Enzyme (2U)	0.2	0.1	0.1
H_2O	up to 50	up to 50	up to 50
Total	50	50	50

The tubes are then incubated at 37°C overnight. The samples then need to be desalted and resuspended in formamide or stored at -20°C until ready to desalt.

Desalting digested PCR products: (this is necessary to ensure adequate loading of fragments onto capillary as automated sequencer loads using an electric current).

1. Digests are desalted by adding 0.25 μL of glycogen (20 mg/ml – Roche), 1/10 volume of 3 M sodium acetate (pH 5.2) and mixing gently. 2.5 volumes of ice-cold ethanol (95%) are then added and tubes well mixed.
2. Note the expected position of the DNA pellet in the tube by positioning the cap hinge toward the outside of the centrifuge rotor. Tubes are centrifuged at 16,000 x g for 15 min at 4 °C.
3. Remove the supernatant being careful not to dislodge the pellet (it may be invisible!)
4. Carefully rinse the pellet twice with 200 μL of ice-cold 70% ethanol while centrifuging at 16,000 x g for 2 min at 4 °C between each wash.
5. Dry the pellet for ~5 min in a vacuum desiccator.
6. Resuspend the pellet in 10 μL deionised formamide (this must be fresh and stored at -20°C).

7. You can store your samples at -20°C for a short period of time before analyzing. If you anticipate that you will not run your samples within the next 8 weeks, then store at -80°C.

Sample preparation for TRFLP electrophoresis.

8. Mix 10 µL of de-ionised formamide with 0.25 µL of size standard (GeneScan Rox-500) per sample (make a master mix first). Pipette master mix into 96-well plates and add 1 µl of sample (digested PCR product) to each well required (in multiples of 16 – sequencer has 16 capillaries). Cover plate with microplate film, ensuring all wells are properly sealed.
9. Just before you run samples on the sequencer you must denature the double stranded DNA by heating at 95°C for 5 min in thermocycler. Place on ice immediately afterwards.
10. Remove plastic film and replace with rubber sequencer gasket. Make sure gasket fits into each well.
11. Now bring plate into 305 Mulford (Garbelloto Lab) on ice and fit to plastic housing frame for sequencer first. Then load into sequencer (ask Amy or myself how to do this) and run in GeneScan mode using default parameters except for RunModule – for this use EoinRunModule. This is the same as default except for 60s injection time compared with 22s in default.