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 25 µl volumes containing

Component	**Volume (µL) for 1 sample**	**Mastermix for 96 samples (µL) (make enough for 100 to allow for loss)**
10X Takara Ex Taq PCR buffer w/MgCL2	2.5	250
Forward primer (3µM)	2.5	250
Reverse primer (3µM)	2.5	250
BSA (5 mg/ml)	2.5	250
dNTP mix Takara (2.5mM)	2	200
Takara ExTaq DNA polymerase (5 U/µL)	0.1	10
ddH_2O	12.4	1240
DNA template	0.5	-
	25	2450 (24.5 µl per well)

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 (350 ml 1x TAE, 3.5g agarose) on large gel box and then purified using a MoBio 96 well PCR product purification kit and vacuum manifold according to manufacturer's instructions and eluted in a final volume of 100 µL.

Restriction digestion:

The purified PCR product is then digested enzymatically in a 96 well PCR plate as follows:

Component	AluI digest (µl)	MspI digest (µl)	HhaI digest (µl)
PCR product (25 µl reaction eluted in 100 µl from 96 well kit)	30	30	30
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	14.8	14.9	14.4
Total	50	50	50

As usual make a master mix with enough reagents for 100 reactions.

The plate is then sealed and incubated in a water bath (or PCR machine) at 37^{o}C overnight. The samples are then desalted and resuspended in formamide or stored at -20^{o}C until ready to desalt.

Desalting digested PCR products: (this is necessary to prevent a noisy baseline on your electropherogram when using an automated sequencer).

Prepare 125mM EDTA ahead of time: dissolve into 18MΩ water and filter sterilize. EDTA takes about 1hr to dissolve. EDTA can be dispensed into sterile boats or 0.2ml tubes for multi-channel pipetting, but do not save used aliquots.

1. Add 1/10 vol 125mM EDTA to each well. Make sure EDTA dissolves into samples. Mix with pipette tips.
2. Add 1/10 vol 3M NaOAc (pH 5.2) to each well. Make sure NaOAc dissolves into samples. Mix with pipette tips. *NaOAc should be prepared regularly, but need not necessarily be made fresh.
3. Add 2.5 vol 100% EtOH (from freezer) to each well. Mix with pipette tips.

4. Replace sealing tape, seal very well and vortex lightly. Incubate at room temp for 15min.

5. Spin @ 2500g 30min.

6. Immediately flick plate contents into sink and lightly tap dry on paper towels.
   *if you do not immediately empty plate once spin has finished (5min or less), give samples a quick spin (2500g, 2min) to re-precipitate pellet

7. Invert plate onto paper towel and spin 185g for 1min to dry.
   *This is to remove any excess NaOAc, which is a critical step. Start timer immediately.

8. Add 35µl 70% EtOH to each well and mix with pipette tips.
   *make EtOH fresh to ensure accurate concentration.

9. Spin @ 2500g, 15min

10. Flick plate contents into sink, invert plate onto paper towel and spin 185g for 1min to dry.
    *start timer immediately.

11. Resuspend samples in 10µl formamide (HiDi). Cover plate with sealing tape, vortex thoroughly (15sec) (spin?) and denature in thermocycler (5min@95°C, 4°C for a minimum of 3min).
    *plates waiting to be run may be stored at –20°C, but probably should be re-denatured before running on the genetic analyzer. Stored plates should be wrapped in foil to protect dyes from light.

Sample preparation for TRFLP electrophoresis.

1. 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 GeneAmp 96-well plates and add 4 µl of sample (digested PCR product in

formamide) to each well required (in multiples of 16 – sequencer has 16 capillaries so add 10 µl formamide to any empty wells). Cover plate with microplate film, ensuring all wells are properly sealed.

2. Just before you run samples on the sequencer you must denature the double stranded DNA by heating at 95^{o}C for 5 min in thermocycler, cool to 4^{o}C for 3 min minimum and then place on ice immediately.
3. Remove plastic film and replace with rubber sequencer gasket. Make sure gasket fits into each well.
4. Now bring plate to Garbelloto Lab (303 Mulford Hall) 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.