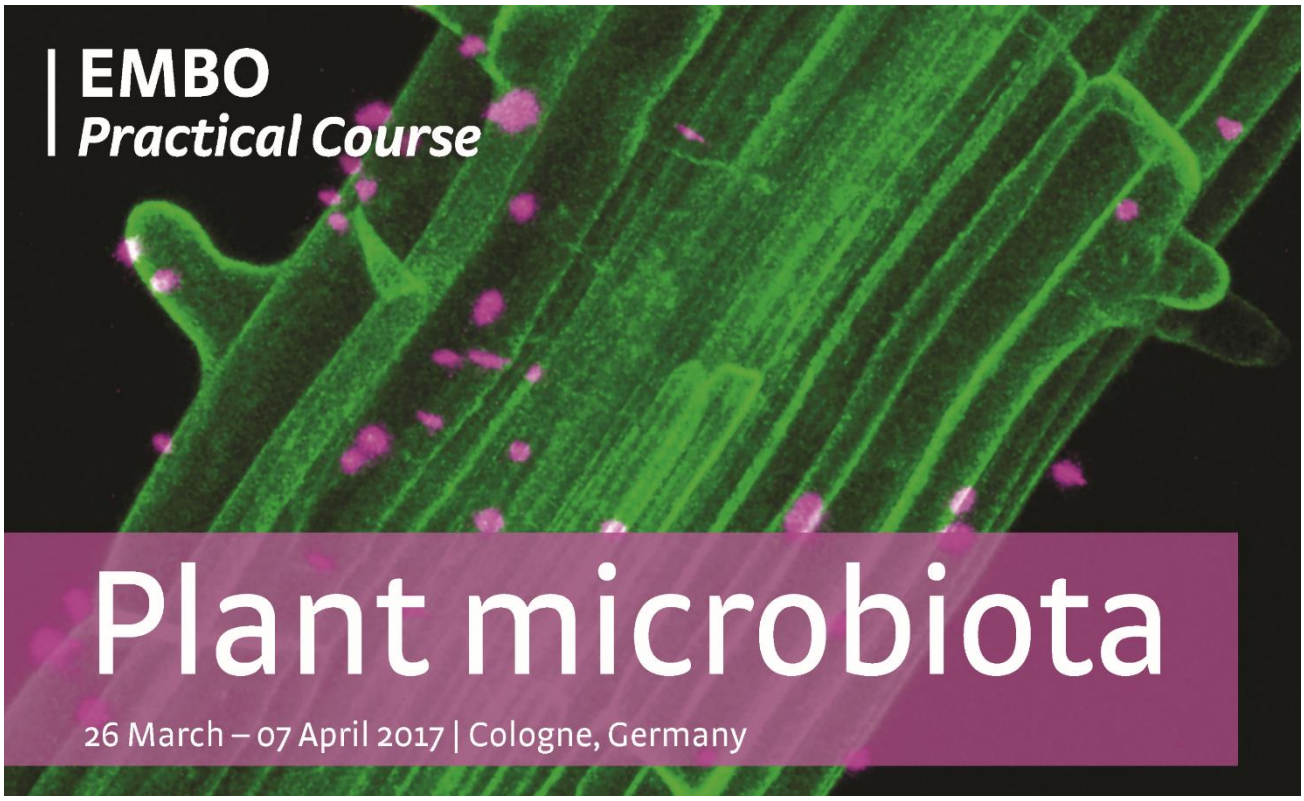

PROTOCOLS

(Week 1)

Fractionation protocol

MATERIALS (for one pot, 4 roots)

- 3% bleach (small volume for root sterilization: 20 ml and large volume for filter holders sterilization: 100 ml)
- 80% ethanol (small volume for root sterilization: 20 ml and large volume for filter holders sterilization: 100 ml)
- Autoclaved water (100 ml)
- Sterile 25mm filter holders (1)
- Sterile 25mm 0.2 microm pore size filter membrane (1)
- 10 or 20 mL sterile syringes (1)
- Detergent (1x TE + 0.1% Triton X-100) (50 ml)
- 15mL falcons (10)
- 50 mL falcon (1)
- 2mL Eppi tubes with screw-lid (Violet tube with beads from DNA isolation kit)
- Sterile blades and tweezers
- Squared petri dish with a 4cm line drawn on it (1)

PROCEDURE

1. Harvest in an Eppi tube a small aliquot of the soil from an unplanted pot or from soil surrounding the plant, being this the **BULK SOIL FRACTION**.

2. Pool 4 roots together, shake them before keeping them in a 15 mL falcon tube, already filled up with autoclaved water (around 10 mL). These roots should still have some soil particles attached to them. Shake the tube 10x and transfer the roots to another 15 mL falcon with 10 mL autoclaved water. Centrifuge the falcon of the first wash (4,000 x g, 15 min), remove 95% of the supernatant and resuspend the pellet into the remaining liquid using a cut P1000 tip. Transfer 300 µl of suspension to a 2 ml tube with lysis matrix (**RHIZOSPHERE FRACTION**).

3. Wash the roots 3 times by inverting 10 times. Change the water every time.

4. Transfer the roots to a new falcon tube, containing 10 mL of detergent. Shake vigorously for 2 min. Keep the wash and transfer the roots to another tube with detergent, and shake again for 2 min. If desired, this can be done a third time to make sure to remove all the microbes tightly attached to the root. Pour the three detergent washes together in a 50 ml falcon tube and transfer the roots to another 15 ml tube.

5. (Before using them, the filter holders should be sterilized by putting them in a beaker and first pouring 3% bleach over them, removing it for later use and, then, pouring 80% ethanol. Remove the ethanol back, cover with miracloth and allow to dry. In between experiments, a UV treatment (for approx. 30 min) can also be done.)

6. Place a filter membrane on the center of a filter holder, trying not to touch it (place it using the blue paper that comes with the membrane). Close the filter holder and attach a 10 ml syringe (without the plunger).
7. Pour the detergent wash into the syringe and push with the plunger into the membrane (It should be hard). Repeat this until all the detergent wash has gone through the membrane.
8. Discard the flow through and keep the membrane, containing the **RHIZOPLANE FRACTION**.
9. Pour 10 mL of 80% ethanol into the tube where the roots were placed and shake for 30 seconds. Discard the ethanol.
10. Repeat the same procedure, with 3% bleach and shake for 30 seconds.
11. Wash 3 times with autoclaved water to remove all the bleach. Cut a 3 cm segment 1 cm below the hypocotyl and keep this root part in an Eppi tube as the **ENDOPHYTIC FRACTION**. Store all fractions in -80 freezer.

DNA isolation from bulk soil and root-associated fractions using the FastDNA Spin kit for Soil (MP Biomedicals)

1. Per sample, label 2x 2 ml tubes, 1x 1.5 ml tube and 1x Spin filter.
2. Switch on the FastPrep Instrument and Thermomixer (55^{o}C) and defrost nuclease-free water.
3. Take the samples from the -80 freezer and transfer to liquid nitrogen.
4. Go the basement of building H with big tweezers, P1000, P100, a rack, sodium phosphate buffer (provided in kit), MT buffer (provided in kit), gloves and tips for P1000 and P200.
5. Set the program to 6200 rpm / 1x 30 seconds.
6. Place the root samples in the FastPrep instrument, put the white lid on top, close the cap and start the instrument.
7. Transfer the samples to the rack and the other samples. Add 978 µl sodium phosphate and 122 µl MT buffer to each tube.
8. Load the tubes to the FastPrep instrument and start homogenization again (same parameters).
9. In the lab: centrifuge all samples (15 min – 14000 x g).
10. Meanwhile pipet 250 µl PPS in 2 ml tubes and transfer after centrifugation 900 µl supernatant to these tubes. Mix 10x by inverting.
11. Centrifuge tubes (5 min – 14000 x g). Meanwhile resuspend Binding matrix by shaking the bottle and pipet 900 µl in a fresh 2 ml tube.
12. After centrifugation, transfer 900 µl supernatant to the tube with Binding matrix. Place on a rotator for 3 min (at about 35 rpm).
13. Put the tubes in a rack for 3 min to allow the matrix to settle.
14. Remove and discard 2x 550 µl supernatant and with the same tip mix the matrix with the remaining supernatant.
15. Pipet the resuspended matrix on a Spin filter and centrifuge (1 min – 14000 x g).
16. Empty the catch tube and add 500 µl SEWS-M (with added EtOH) and resuspend the matrix. Centrifuge (1 min – 14000 x g). Empty the catch tube.
17. Spin matrix/filter to dry (2 min – 14000 x g).
18. Place the Spin filter in a new 1.5 ml tube and add 50 µl of nuclease-free water. Mix the matrix by stirring with the tip (no pipetting!).
19. Close the filter and incubate in the Thermomixer for 5 min (55^{o}C).
20. Centrifuge (2 min – 14000 x g) to elute the DNA.
21. Store in the fridge (short-term storage) or freezer (long-term storage).

Library preparation PCR

Preparation template DNA

Determine the DNA concentration of the template using PicoGreen method (see protocol)

Dilute the template DNA to 3.5 ng/µl if necessary (24 µl total volume)

Sample name	Conc (ng/µl)	Sample	nuclease-free H2O
Example	7	6	6
		=3,5/Conc*24	=24-Sample

PCR1

Primers

F-primer (name, TM)	799F	Bacteria
R-primer (name, TM)	1192R	
amplicon (bp)	1 band, ~400bp	

F-primer (name, TM)	ITS1F	Fungi
R-primer (name, TM)	ITS2	
amplicon (bp)	1 band, ~500bp	

Platelayout

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Soil 1	RSph 1	RPla 1	Root 1									Bacteria
B	Soil 1	RSph 1	RPla 1	Root 1									Bacteria
C	Soil 1	RSph 1	RPla 1	Root 1									Bacteria
D	-	-	-	-									Bacteria
E	Soil 1	RSph 1	RPla 1	Root 1									Fungi
F	Soil 1	RSph 1	RPla 1	Root 1									Fungi
G	Soil 1	RSph 1	RPla 1	Root 1									Fungi
H	-	-	-	-									Fungi

Handling

1) Prepare 2 MM (Bacteria, Fungi). Add 88 µl MM bacteria into row A (A1 to A4) and 88 µl MM fungi into row E (E1 to E4) in a PCR plate

2) Add 3 µl H20 into D and H

3) Distribute 22 µl of MM A and E into D and H

4) Add 9 µl DNA to A and E

5) Distribute 25 µl to B-C and F-G

Master Mix (MM)

Bacteria	[final]	reaction 1x	MM _x 4.1	Number of samples 5.0
Incomplete buffer (10x)	1x	2.5	10.25	51.25
MgCl2		0.5	2.05	10.25
BSA 3%		2.5	10.25	51.25
dNTPs (10mM each)	200 µM	0.5	2.05	10.25
F-primer 799F (10µM)	300 nM	0.75	3.075	15.375
R-primer 1192R (10µM)	300 nM	0.75	3.075	15.375
BIORON DFS-Taq	2 U	0.4	1.64	8.2
H2O		14.1	57.81	289.05
MM volume (-template)		22	90.2	451
DNA Template (3.5 ng/ul)		3		
Final volume:		25		

Fungi	[final]	reaction 1x	MM __x 4.1	Number of samples 5.0
Incomplete buffer (10x)	1x	2.5	10.25	51.25
MgCl2		0.5	2.05	10.25
BSA 3%		2.5	10.25	51.25
dNTPs (10mM each)	200 µM	0.5	2.05	10.25
F-primer ITS1F (10µM)	300 nM	0.75	3.075	15.375
R-primer ITS2 (10µM)	300 nM	0.75	3.075	15.375
BIORON DFS-Taq	2 U	0.4	1.64	8.2
H2O		14.1	57.81	289.05
MM volume (-template)		22	90.2	451
DNA Template (3.5 ng/ul)		3		
Final volume:		25		

PCR program thermocycler

Step	temp (°C)	time	
1	94	2'	
2	94	30''	25 x
3	55	30''	
4	72	60''	
5	72	10'	
6	4	∞	

PCR1 Cleanup

1) Pool the 3 replicates together in A (Bacteria) and E (Fungi) and keep the control in D and H (16 samples for digestion)

2) Prepare MM without PCR product on ice

	1 reaction	17 reactions
Antarctic phosphatase:	1 µL	17 µl
Exo I:	1 µL	17 µl
Ant. Phosph. Buffer:	2,44 µL	41,48 µl

3) Add 4,44 µl of MM to a clean PCR plate in the same layout as PCR 1 (row A, D, E and H)

4) Add 20 µl of PCR1 product in the corresponding well

5) Incubation program thermocycler

1. Incubate reaction at 37°C for 30 minutes (prepare MM PCR2)
2. Deactivate the enzymes at 85°C for 15 minutes
3. Centrifuge 10 minutes at 3000 rpm and transfer supernatant afterwards to new plate
4. Only transfer 12 µl of supernatant as BSA precipitates and easily clogs the pipett!!!!
5. Store at 4°C until use (this is the template for the second PCR reaction)

PCR2

Platelayout

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Soil 1 B5-1	RSph 1 B5-2	RPla 1 B5-3	Root 1 B5-4									Bacteria
B	Soil 1 B5-1	RSph 1 B5-2	RPla 1 B5-3	Root 1 B5-4									Bacteria
C	Soil 1 B5-1	RSph 1 B5-2	RPla 1 B5-3	Root 1 B5-4									Bacteria
D	-	-	-	-									Bacteria
E	Soil 1 Ft-1	RSph 1 Ft-2	RPla 1 Ft-3	Root 1 Ft-4									Fungi
F	Soil 1 Ft-1	RSph 1 Ft-2	RPla 1 Ft-3	Root 1 Ft-4									Fungi
G	Soil 1 Ft-1	RSph 1 Ft-2	RPla 1 Ft-3	Root 1 Ft-4									Fungi
H	-	-	-	-									Fungi

Handling

1) Prepare 2 MM (Bacteria, Fungi) with forward primers, distribute 85 µl of MM to row A (Bacteria) and E (Fungi) of a PCR plate

2) Add 3 µl Reverse primers with barecodes individually in row A and E (4 barcoded reverse primers for bacteria, 4 barcoded reverse primers for fungi)

3) Add 3 ul of the digested control product to row D and H

4) Distribute 22 ul of MM to D and H

5) Add 9 µl of digested PCR product to A (Bacteria) and E (Fungi)

6) Distribute 25 µl to B-C and F-G

Master Mix (MM)

Bacteria	[final]	reaction 1x	MM __x 4.1	number of samples 5.0
Incomplete buffer (10x)	1x	2.5	10.25	51.25
MgCl2		0.5	2.05	10.25
BSA 3%		2.5	10.25	51.25
dNTPs (10mM each)	200 µM	0.5	2.05	10.25
F-primer B5-F (10µM)	300 nM	0.75	3.075	15.375
R-primer (10µM)	300 nM	0.75		
BIORON DFS-Taq	2 U	0.4	1.64	8.2
H2O		14.1	57.81	289.05
MM volume (-template)		22	87.125	435.625
DNA Template (from Clean-up)		3		
Final volume:		25		

Fungi	[final]	reaction 1x	MM __x 4.1	number of samples 5.0
Incomplete buffer (10x)	1x	2.5	10.25	51.25
MgCl2		0.5	2.05	10.25
BSA 3%		2.5	10.25	51.25
dNTPs (10mM each)	200 µM	0.5	2.05	10.25
F-primer Fm-F (10µM)	300 nM	0.75	3.075	15.375
R-primer (10µM)	300 nM	0.75		
BIORON DFS-Taq	2 U	0.4	1.64	8.2
H2O		14.1	57.81	289.05
MM volume (-template)		22	87.125	435.625
DNA Template (from Clean-up)		3		
Final volume:		25		

PCR program thermocycler

Step	temp (°C)	time	
1	94	2'	
2	94	30''	10 x
3	55	30''	
4	72	60''	
5	72	10'	
6	4	∞	

After PCR 2, pool the 3 replicates together in A (Bacteria) and E (Fungi).

Agarose gel: Transfer 7.5 µl of PCR products and in 2.5 µl of loading dye (Orange G).

PCR product purification (AMPure): use 50 µl of PCR product.

Purification – AMPure

Procedure

- Prepare 1 ml fresh (!) 70% EtOH by combining 700 µL 100% EtOH with 300 µl nuclease-free water.
- Pool the 3 replicates together in A (Bacteria) and E (Fungi).
- Transfer 50 µl PCR product to a fresh 1.5 ml tubes. Resuspend the AMPure XP beads and transfer 50 µl bead (1) suspension to the tubes. Mix thoroughly by pipetting 10 times. Incubate for 5 min at room temperature (2).
- Place the tubes in a magnetic rack for 3 min to separate the beads from the solution (3).
- Remove and discard the cleared solution by pipetting. Add 500 µl of 70% EtOH and incubate for 1 min (4). Remove the EtOH and repeat the washing step with 70% EtOH (4). Dry the beads for 5 min (do not overdry since this will decrease the elution efficiency).
- Remove the tubes from the rack. Add 50 µl (or less if you want to concentrate) elution buffer or nuclease-free water to the tube and mix by pipetting 10 times (5).
- Centrifuge (1 min - 14000 x g) and place the tubes in the magnetic rack.
- Transfer the eluent to a fresh 1.5 ml tube (6).

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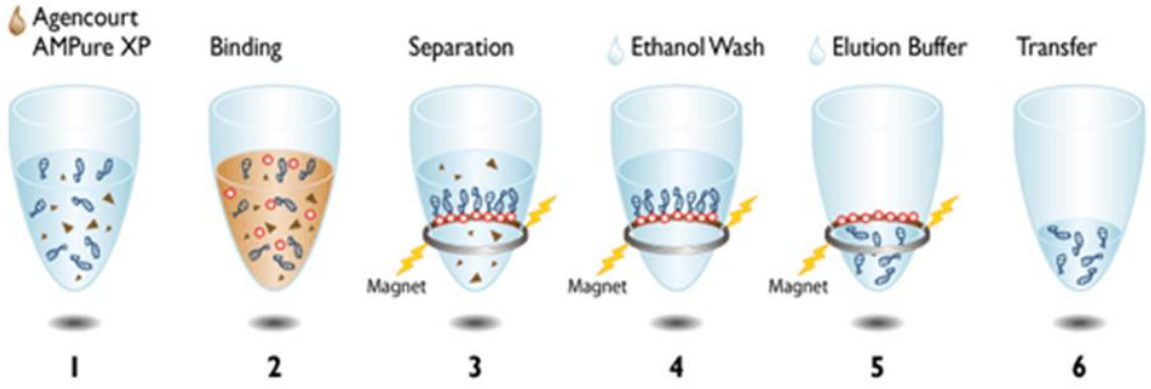

Measure of DNA concentration using Picogreen

- Prepare a dilution series (standard curve): 20 ng/µl, 10, 5, 1, 0.5, TE in 1x TE buffer.
- Dilute 200x picogreen in TE 1x (pico working solution).

sample/std. volume	**4 µl**		
# samples	**8**	12 std	26.4
Pico working solution	**40 µl**	1:200	**1056 (40x26.4)**
Pico stock	**5.28 (1056/200)**		
1x TE	**1050.72**		

TE working solution	1200		
20XTE stock	60	**H20**	1140

- Add 40 µl of pico working solution in wells of a qPCR plate.
- Add 4 µl of sample or 4 µl of the dilution series.
- Run qPCR (1 cycle @25°C 1 min, 3 cycles @25°C 30 seconds) – fluorescence measurement at end of each the three cycles).
- Substract TE fluorescence background.
- Plot standard curve.
- Divide sample fluorescence intensity by slope of standard curve.
- See excel spreadsheet for DNA concentration calculation.

Measure of DNA concentration using Qubit

Prepare dye and 1x TE buffer

- Put in 2 2ml tubes 1900µl nuclease free H2O
- Add in both 100µl 20x TE buffer
- Of one tube take out 10µl and add therefore 10µl dye

Prepare samples

- Blank: in 0,5ml PCR tube add 100µl of prepared dye
  add 100µl 1x TE buffer
- Standard DNA 100 ng/µl: add in 0,5ml PCR tube 100µl of prepared dye
  add 98µl 1x TE buffer
  add 2µl standard DNA
- Samples: see above (standard DNA)
  add 2µl of sample
- Mix tubes and incubate for five minutes in the dark

- measure Blank and Standard DNA and then samples

Pooling libraries together

- Pool libraries with a concentration of 200 ng/µl together
- Dilute final pool 1:2, 1:10, 1;100
- Measure final pool and dilution