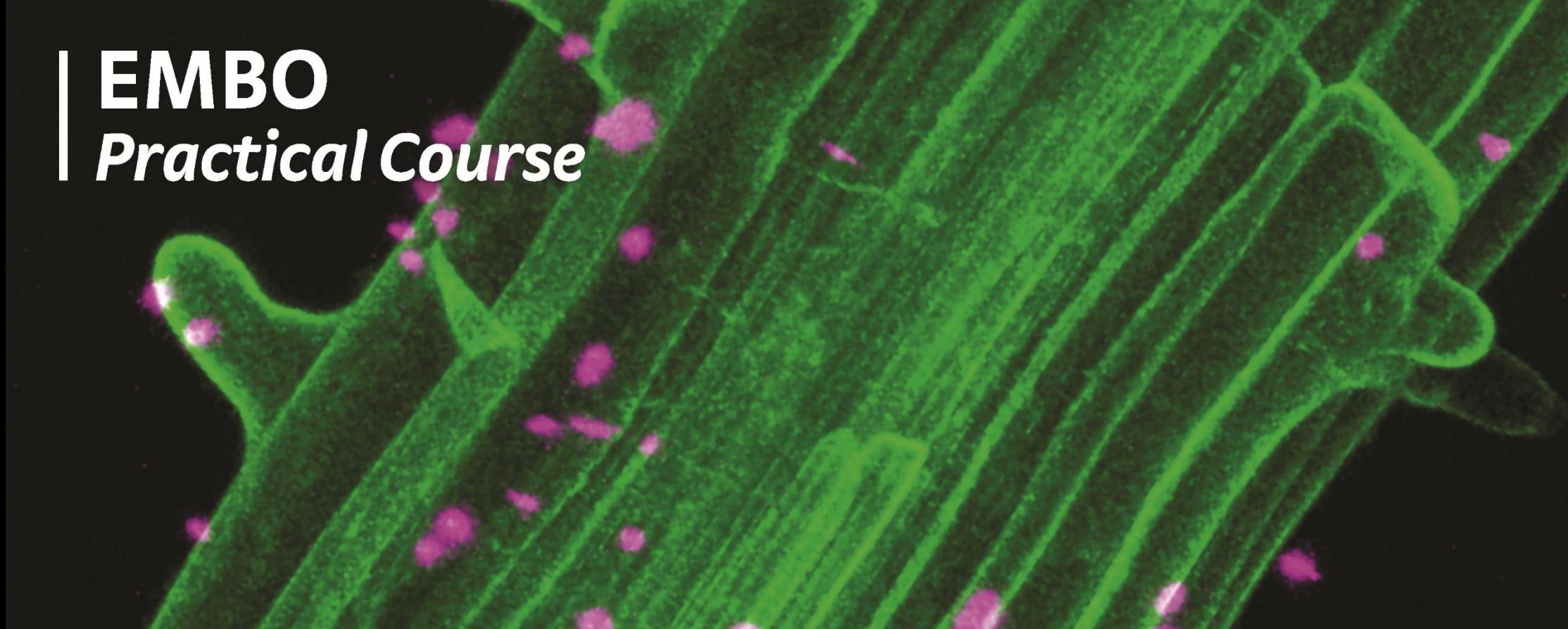


| EMBO
| *Practical Course*

A fluorescence microscopy image showing plant leaves with distinct green and pink fluorescent signals. The green signal appears as bright, parallel lines running along the length of the leaves, likely representing the vascular system or specific cellular structures. The pink signal is more scattered and punctate, appearing as small, bright spots across the leaf surface, possibly representing different microbial species or cellular components. The background is dark, making the fluorescent signals stand out.

Plant microbiota

26 March – 07 April 2017 | Cologne, Germany

Day 1 – Sunday March 26th

Arrival

Day 2 – Monday March 27th

08:20 – 08:30

Registration at SR 1

Keynote session: The structure of the plant microbiome SR 1

08:30 - 09:00

Opening remarks - **Paul Schulze-Lefert**

09:00 - 10:00

Keynote lecture "*Niche adaptation of the Arabidopsis leaf microbiota*" - **Julia Vorholt** LH

10:00 - 11:00

Lecture "*Microbial interactions at the plant root interface*" - **Stéphane Hacquard** LH

11:00 - 11:15

Coffee break SR 1

11:15 - 11:45

Safety training by Diana Hofmann SR 1

11:45 - 13:00

Introduction to the practical afternoon session

13:00 - 14:00

Lunch C

Afternoon session: Sample preparation for high-throughput profiling lab

14:00 - 16:30

Part I: Plant harvest and fractionation

16:30 - 16:45

Coffee break C

16:45 - 19:00

Part II: DNA isolation

19:00 - 21:00

Dinner C

Culture-independent approach (week 1)

Sample preparation,
fractionation, DNA isolation



Library preparation
(ITS, 16s rRNA)



MiSeq



Data analysis (sample
demultiplexing, OTU
clustering, OTU table,
diversity indices, PcoA...)

Culture-dependent approach, microbiota reconstitution experiments and genome/metagenome analysis (week 2)

Sample preparation
and fractionation



Establishment of bacterial
and fungal culture collections



SynCom experiments using
gnotobiotic plant systems
(Calcined clay and FlowPot
systems)



Analysis of SynCom data

Identification of isolates
Taxonomic classification,
estimation of recovery
rates



Whole genome sequencing and
comparative genome analysis



Measuring selective
pressure



Day 2 – Monday March 27th

Sample preparation

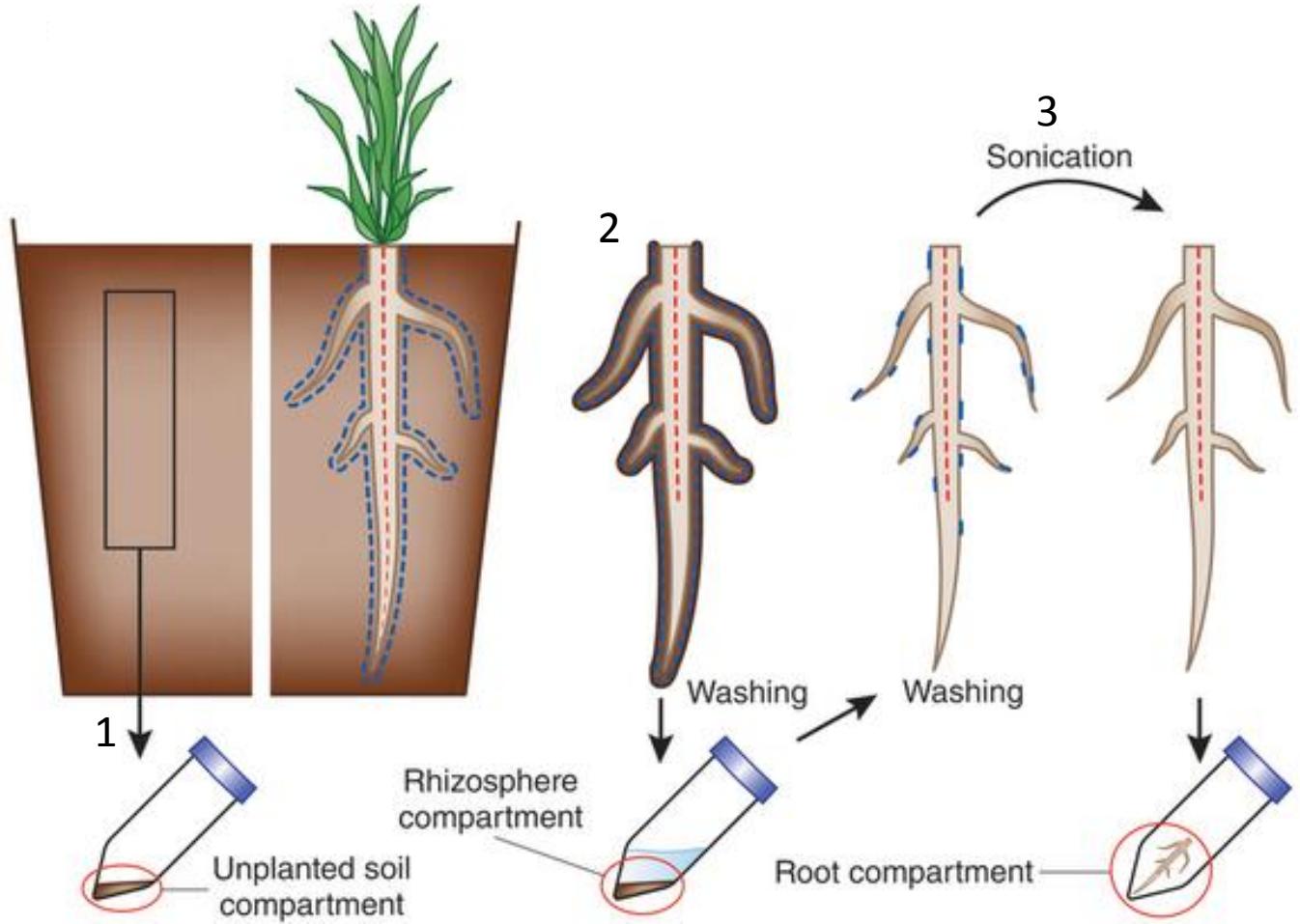
Fractionation, DNA isolation, picogreen

Day 2 – Monday March 27th

Fractionation into 3 compartments

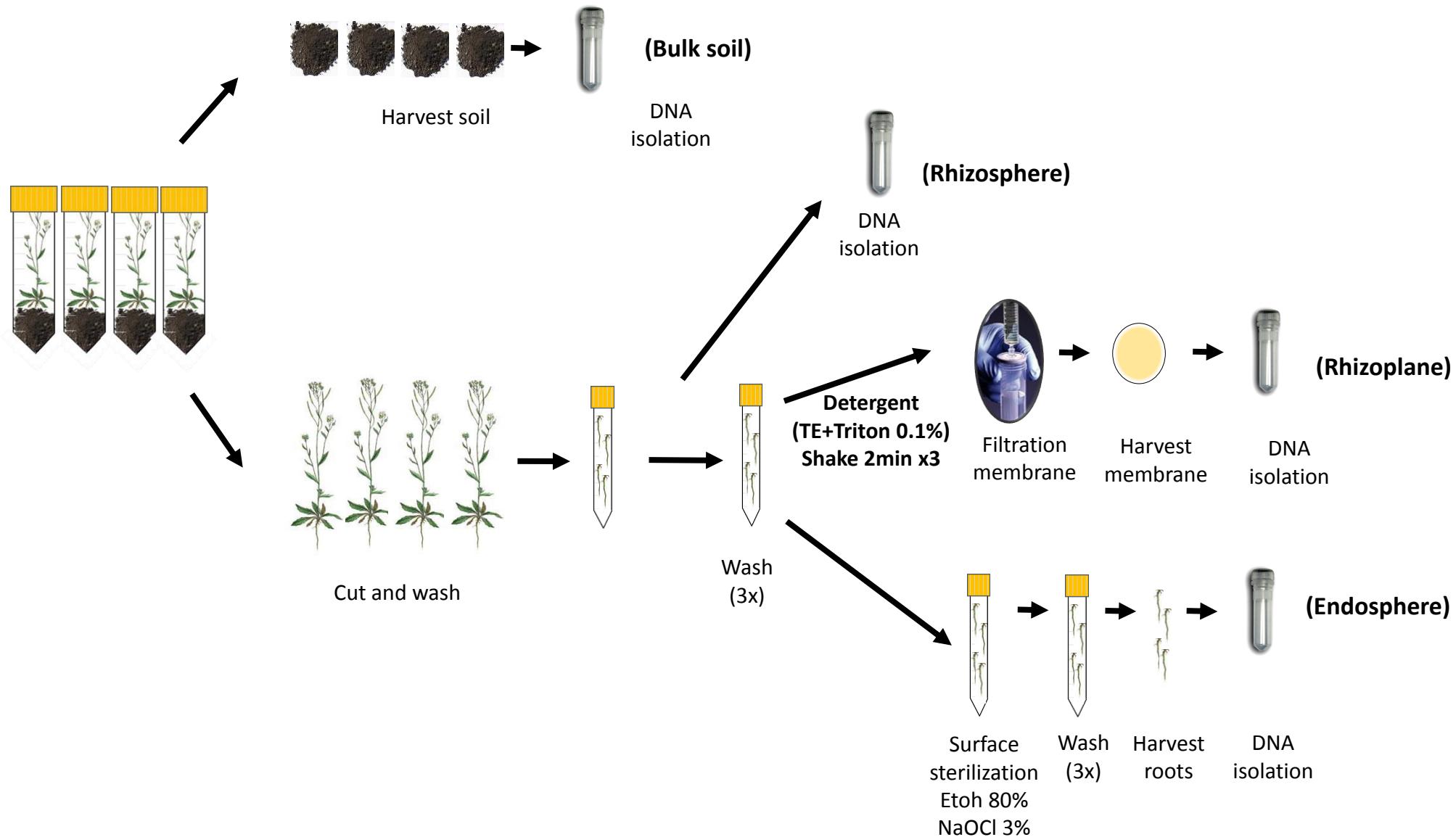


1. Direct sampling (pooled subsamples) of bulk soil preferable from pots/plots without plants (**BULK SOIL**) into a tube with lysis matrix.
2. Dig out root system, remove excessive soil and cut out a 3 cm segment 1 cm under the hypocotyl. Wash in PBS+silwet buffer. Wash again in PBS+S buffer. Spin the first wash to obtain a pellet of soil and transfer the pellet to a tube with lysis matrix (**RHIZOSPHERE**).
3. Transfer root system to fresh PBS+S buffer and sonicate. Wash in fresh PBS+S buffer, dry and transfer to a tube with lysis matrix (**ENDOSPHERE**)



Day 2 – Monday March 27th

Fractionation into 4 compartments adapted for field samples



Day 2 – Monday March 27th

| | ROOT | WASH | SEM IMAGING | |
|--------------------------------------|------|------|-------------|--|
| NON TREATED | | | | |
| DETERGENT 1 | | | | |
| DETERGENT 2 | | | | |
| DETERGENT 3 | | | | |
| SURFACE STERILIZED WITHOUT DETERGENT | | | | |
| SURFACE STERILIZED AFTER DETERGENT | | | | |
| ENDOPHYTIC FRACTION | | | | |

- ✓ Detergent steps successfully remove microbes present on the root surface
- ✓ Surface sterilization is needed but it is not enough by itself
- ✓ Endophytic fraction can be recovered after fractionation protocol

→ Differentiable root fractions
→ Isolation of live microbiota from each fraction possible

Day 2 – Monday March 27th

Root fractionation protocol

MATERIALS

- 3% bleach (small volume for root sterilization and large volume for filter holders sterilization)
- 80% ethanol (same as bleach)
- Autoclaved water
- Sterile 25 mm filter holders
- Sterile 25 mm 0.2 micron pore size filter membranes
- 10 mL sterile syringes
- Detergent (1x TE + 0.1% Triton X-100)
- 15 mL falcons
- 2 mL Eppi tubes with screw-lid
- Sterile blades and tweezers
- Squared petri dish with a 3 cm line drawn on it

Day 2 – Monday March 27th

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 ($4,000 \times g$, 15 min), remove 95% of the supernatant and resuspend the pellet into the remaining liquid using a cut P1000 tip. Transfer 300 μ l 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.

Day 2 – Monday March 27th

DNA isolation protocol

MATERIALS

- 2x 2 ml tubes, 1x 1.5 ml tube and 1x Spin filter
- Buffers
- Samples in liquid nitrogen
- big tweezers, P1000, P100, a rack, sodium phosphate buffer, MT buffer, gloves and tips for P1000 and P200



Day 2 – Monday March 27th

PROCEDURE

1. Take the samples from the -80 freezer and transfer to liquid nitrogen.
2. 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.
3. Set the program to 6200 rpm / 1x 30 seconds.
4. Place the root samples in the FastPrep instrument, put the white lid on top, close the cap and start the instrument.
5. Transfer the root and other samples to the rack and the other samples. Add 978 µl sodium phosphate and 122 µl MT buffer to each tube.
6. Load the tubes to the FastPrep instrument and start homogenization again (same parameters).
7. In the lab: centrifuge all samples (15 min – 14000 x g).
8. Meanwhile pipet 250 µl PPS in 2 ml tubes and transfer after centrifugation 900 µl supernatant to these tubes. Mix 10x by inverting.
9. 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.

Day 2 – Monday March 27th

PROCEDURE

10. After centrifugation, transfer 900 µl supernatant to the tube with Binding matrix. Place on a rotator for 3 min (at about 35 rpm).
11. Put the tubes in a rack for 3 min to allow the matrix to settle.
12. Remove and discard 2x 550 µl supernatant and with the same tip mix the matrix with the remaining supernatant.
13. Pipet the resuspended matrix on a Spin filter and centrifuge (1 min – 14000 x g).
14. 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.
15. Spin matrix/filter to dry (2 min – 14000 x g).
16. 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!).
17. Close the filter and incubate in the Thermomixer for 5 min (55°C).
18. Centrifuge (2 min – 14000 x g) to elute the DNA.
19. Store in the fridge (short-term storage) or freezer (long-term storage).

Day 3 – Tuesday March 28th

Opening session: Symbiotic plant-microbe interactions in a community context LH

09:00 - 10:00 Lecture "Symbiotic nitrogen fixation and its impact on *Lotus japonicus* root-associated microbiota" - **Simona Radutoiu**

Morning session: preparation of sequencing library - part I

10:00 - 10:30 Introduction SR 1

10:30 - 10:45 Coffee break SR 1

10:45 - 13:00 Hands-on: Fungal ITS and bacterial 16S rRNA gene amplification
(PCR 1) lab

13:00 - 14:00 Lunch C

Afternoon session: preparation of sequencing library - part II lab

14:00 - 15:45 Fungal ITS and bacterial 16S rRNA gene amplification (digestion -
PCR 2)

15:45 - 16:00 Coffee break C

16:00 - 19:00 Hands-on: DNA purification

19:00 - 21:00 Dinner C

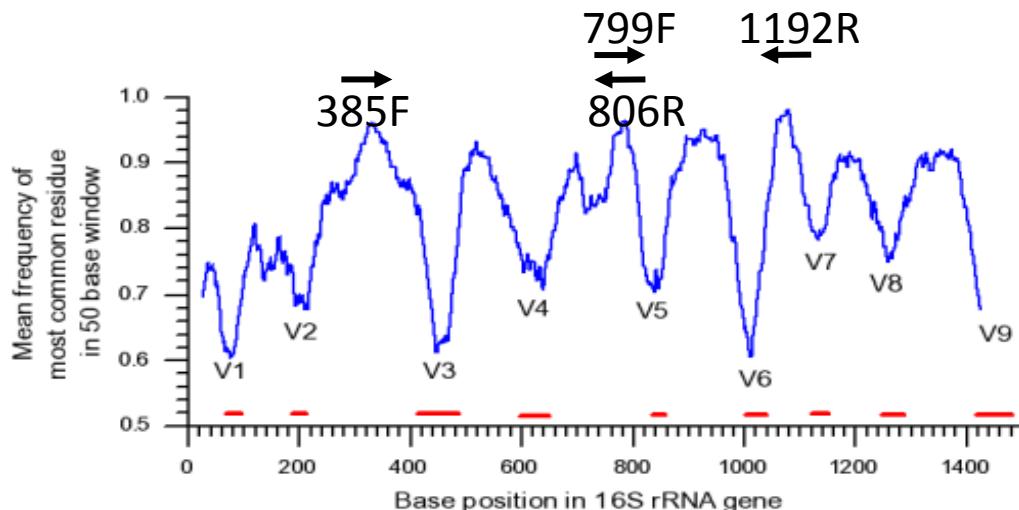
Introduction to the practical session

Preparation of sequencing library (partI)

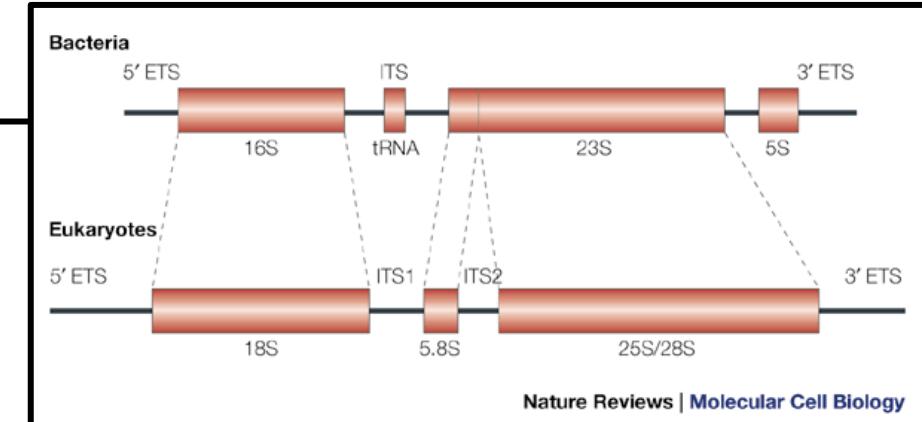
- Primer selection & design
- PCR1
- PCR Clean-Up
- PCR2
- Purification
- Pooling

Day 3 – Tuesday March 28th

Primer selection & design

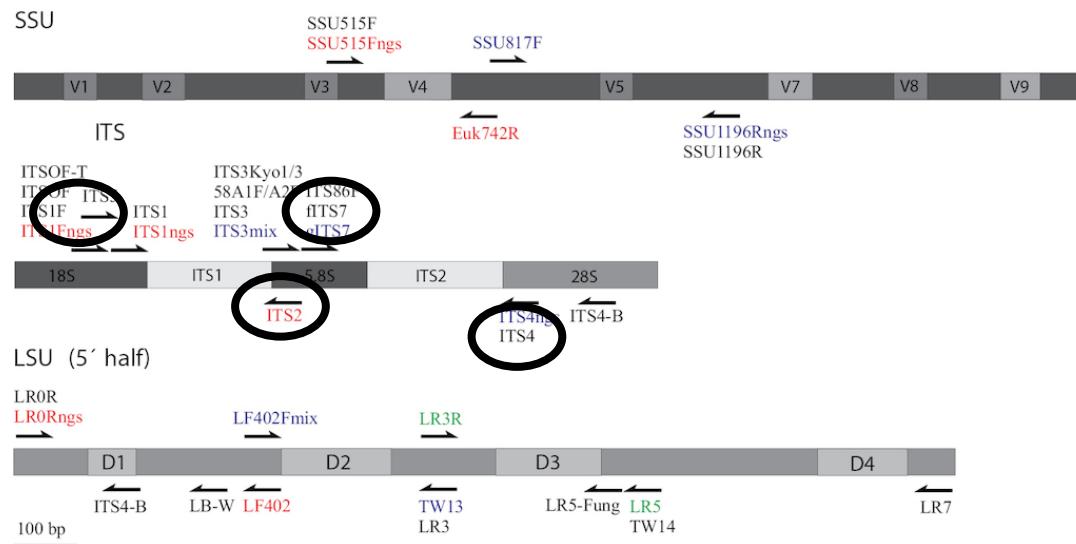


Bacteria:
16S rRNA gene



Primers used at the MPIPZ:
Lundberg et al. 2012 (V3-V4)
Schlaeppi et al. 2013 (V5-V6)

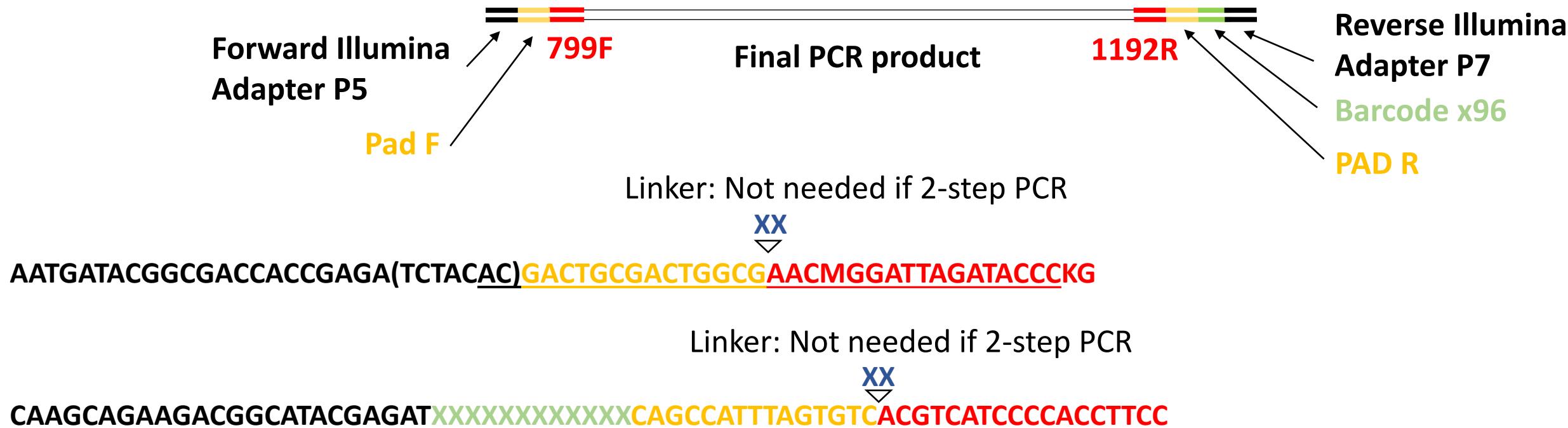
Fungi:
SSU: 18S rRNA gene
ITS
LSU: 25/28S rRNA gene



Primers used at the MPIPZ:
Gardes & Bruns, 1993 (ITS1)
Ihrmark et al. 2012 (ITS2)

Amplicon length: 400-500bp

Day 3 – Tuesday March 28th



Illumina adapters: adapter constructs having flow cell binding sites, P5 and P7, which allow the PCR fragment to attach to the flow cell surface

(TCTACAC): Dual indexing construct

PADs: The pad is a 10-15nt sequence to 1) boost the sequencing primer melting temperature near 65°C and 2) reduce hairpin formation and primer dimers.

Barcode: 12 nt sequence. Must balance the number of bases at each position. 2168 barcodes developed by Golay (see list in Caporaso et al. ISMEJ 2012). Check hairpins, self dimers and cross dimers.

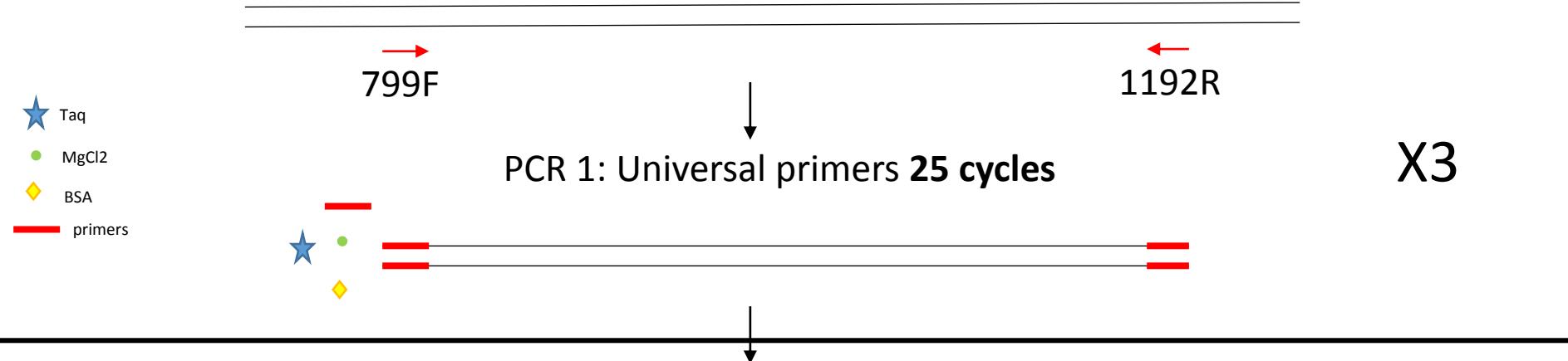
Linker: 2 bp primer linker in 5' end of the amplicon primers to prevent taxon-specific PCR bias due to matches to the PAD

Day 3 – Tuesday March 28th

| | | |
|----------|------------|---|
| Bacteria | PCR1 | |
| | 799F | AACMGGATTAGATAACCCKG |
| | 1192R | ACGTCACTCCCCACCTTCC |
| | PCR2 | |
| | B5-F | AATGATAACGGCGACCACCGAGA(TCTACAC) GACTGCGACTGGCGAACMGGATTAGATAACCCKG |
| | B5-R | CAAGCAGAAGACGGCATACGAGATXXXXXXXXXX CAGCCATTAGTGTACGTCACTCCCCACCTTCC |
| | Sequencing | |
| | B5-R1 | ACGACTGCGACTGGCGAACMGGATTAGATAACC |
| | B5-R2 | CAGCCATTAGTGTACGTCACTCCCCACCTTCC |
| | B5-Index | GGAAGGTGGGGATGACGTGACACTAAATGGCTG |

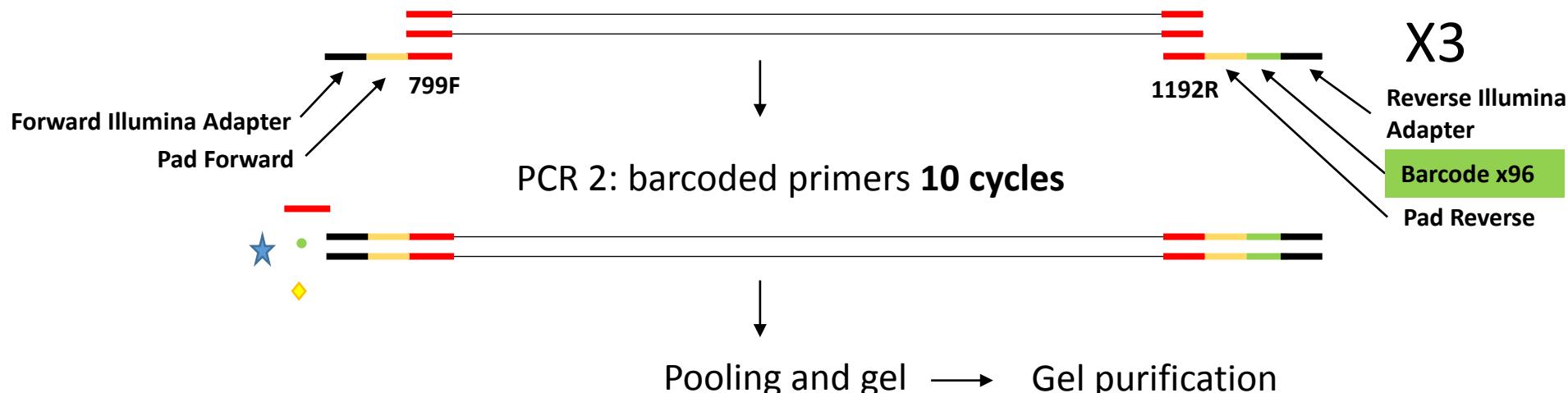
| | | |
|-------|------------|--|
| Fungi | PCR1 | |
| | ITS1F | CTTGGTCATTAGAGGAAGTAA |
| | ITS2 | GCTGCCTTCTTCATCGATGC |
| | PCR2 | |
| | Fm-F | AATGATAACGGCGACCACCGAGA(TCTACAC) TCACGCGCAGG CTTGGTCATTAGAGGAAGTAA |
| | Ft-1 | CAAGCAGAAGACGGCATACGAGATXXXXXXXXXX CGTACTGTGGAGAGCTGCCTTCTTCATCGATGC |
| | Sequencing | |
| | Ft-R1 | TCACGCGCAGG CTTGGTCATTAGAGGAAGTAA |
| | Ft-R2 | CGTACTGTGGAGAGCTGCCTTCTTCATCGATGC |
| | Ft-Index | GCATCGATGAAGAACGCAGCTCTCCACAGTACG |

Day 3 – Tuesday March 28th



Pooling and PCR cleanup: protocol to enzymatically cleanup PCR amplification:
(Antarctic phosphatase, Exo I nuclease)

37°C for 30 minutes
85°C for 15 minutes



Day 3 – Tuesday March 28th

PCR1

Master Mix (MM)

| Bacteria | [final] | reaction | MM _x | Number of samples |
|--------------------------|---------|----------|-------|-------------------|
| | | 1x | 4.1 | 5.0 |
| Incomplete buffer (10x) | 1x | 2.5 | 10.25 | 51.25 |
| MgCl ₂ | | 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 |
| H ₂ O | | 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 | MM _x | Number of samples |
|--------------------------|---------|----------|-------|-------------------|
| | | 1x | 4.1 | 5.0 |
| Incomplete buffer (10x) | 1x | 2.5 | 10.25 | 51.25 |
| MgCl ₂ | | 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 |
| H ₂ O | | 14.1 | 57.81 | 289.05 |
| MM volume (-template) | | 22 | 90.2 | 451 |
| DNA Template (3.5 ng/ul) | | 3 | | |
| Final volume: | | 25 | | |

Plate Layout

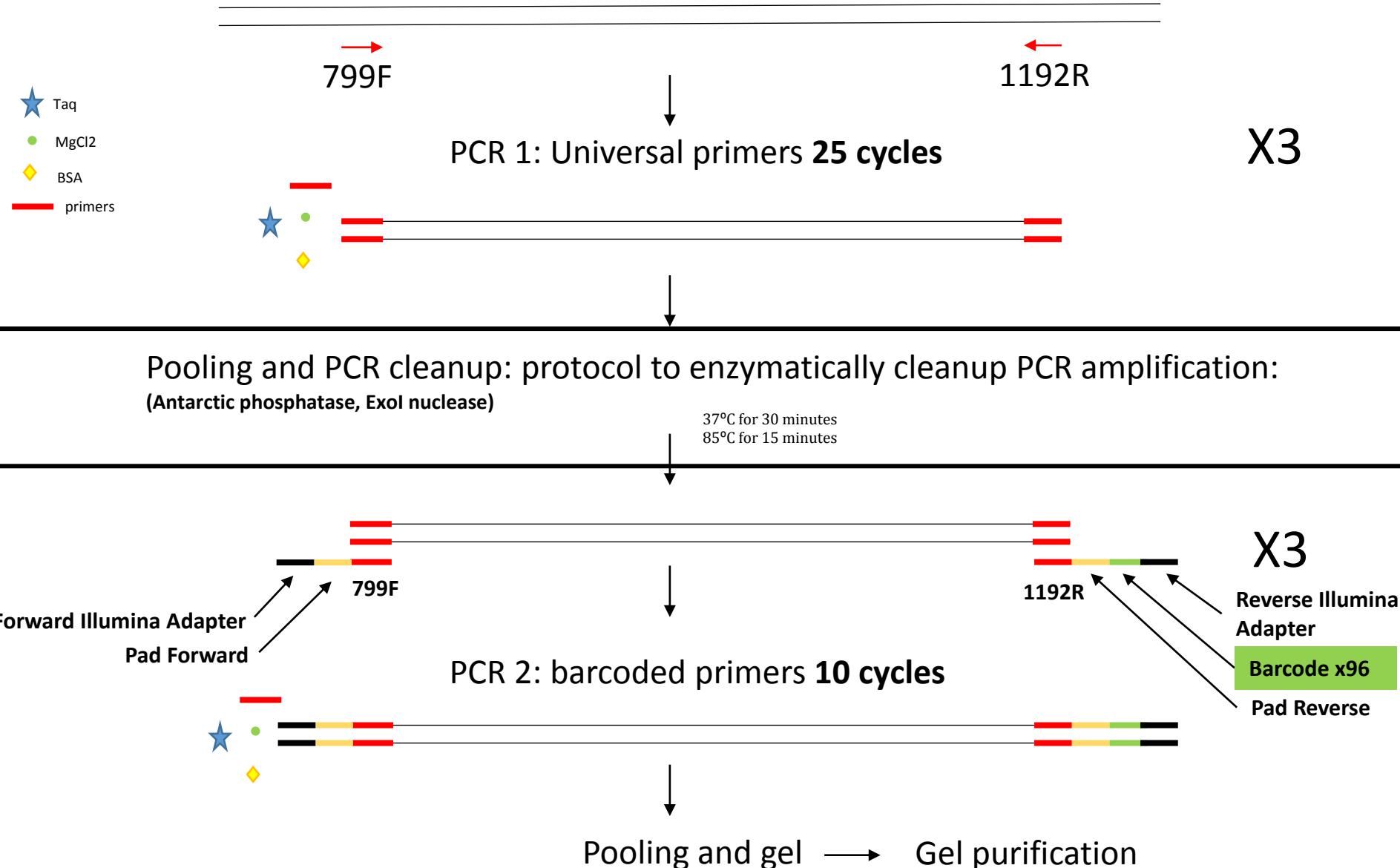
| Platelayout: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|--------------|--------|--------|--------|--------|---|---|---|---|---|----|----|----|----------|
| A | Soil 1 | RSph 1 | RPla 1 | Root 1 | | | | | | | | | Bacteria |
| B | Soil 1 | RSph 1 | RPla 1 | Root 1 | | | | | | | | | Bacteria |
| 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 |

- 1) Add 88 μl MM bacteria into row A (A1 to A4) and 88 μl MM fungi into raw E (E1 to E4) in PCR plate
- 2) Add 3 μl H₂O 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

PCR program thermocycler

| Step | temp ° | time | |
|------|--------|------|------|
| 1 | 94° | 2' | |
| 2 | 94° | 30" | |
| 3 | 55° | 30" | 25 x |
| 4 | 72° | 60" | |
| 5 | 72° | 10' | |
| 6 | 4° | p | |

Day 3 – Tuesday March 28th



Day 3 – Tuesday March 28th

PCR Clean-Up

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

| | 1 reaction | 17 reactions |
|------------------------|------------|-----------------|
| Antarctic phosphatase: | 1 µL | 17 µl |
| ExoI: | 1 µL | 17 µl |
| Ant. Phosph. Buffer: | 2.44 µL | 41.48 µl |

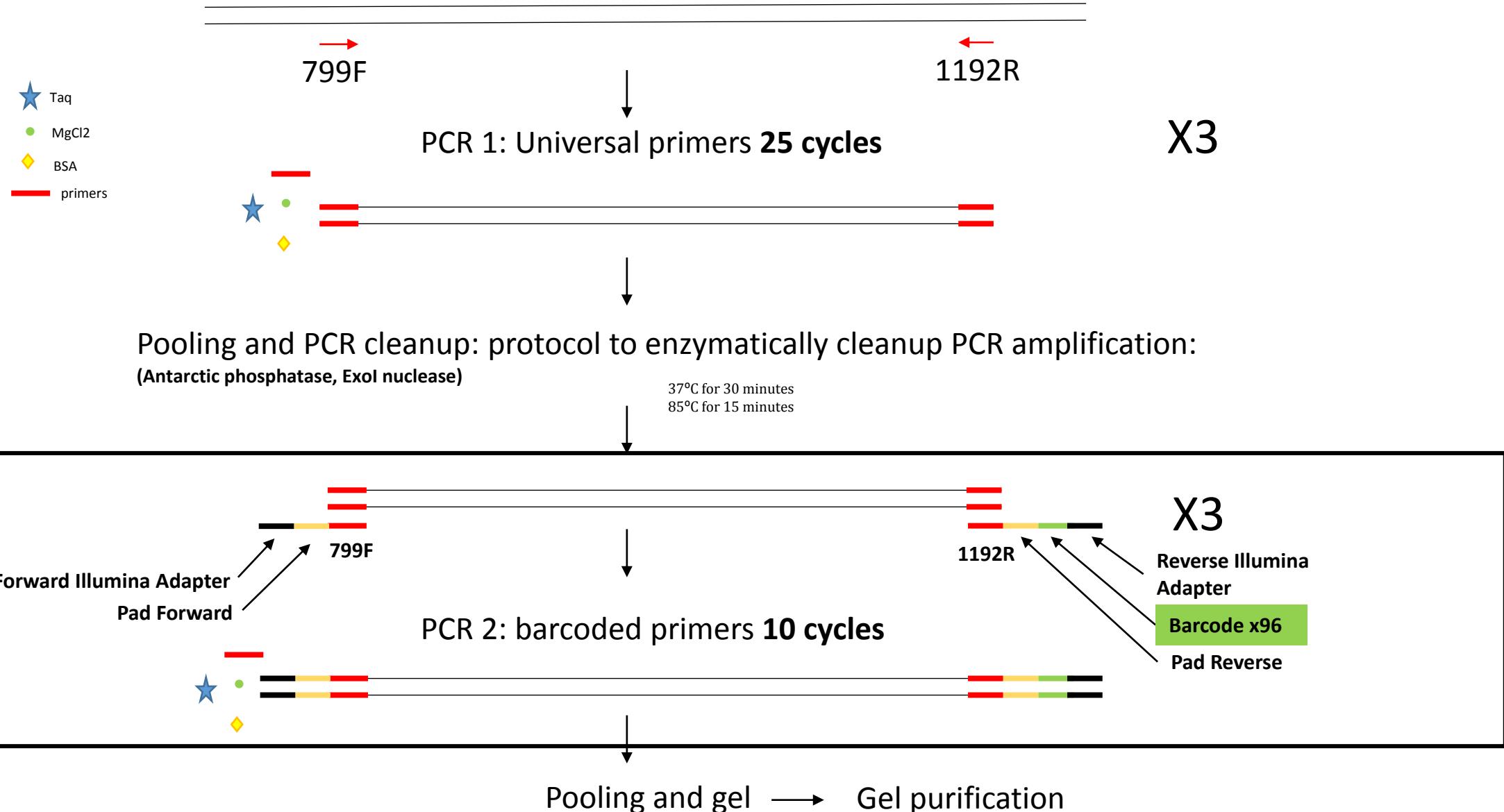
3) Add 4.44 µl of MM to a clean PCR plate in the same layout as PCR 1 (raw A,D,E and H)

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

5) Incubation program thermocycler

1. Incubate reaction at 37°C for 30 minutes
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)

Day 3 – Tuesday March 28th



Day 3 – Tuesday March 28th

PCR2

Master Mix (MM)

| Bacteria | | reaction | MM __x | number of samples |
|------------------------------|---------|----------|--------|-------------------|
| | [final] | 1x | 4.1 | 5.0 |
| Incomplete buffer (10x) | 1x | 2.5 | 10.25 | 51.25 |
| MgCl ₂ | | 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 |
| H ₂ O | | 14.1 | 57.81 | 289.05 |
| MM volume (-template) | | 22 | 87.125 | 435.625 |
| DNA Template (from Clean-up) | | 3 | | |
| Final volume: | | 25 | | |

| Fungi | | reaction | MM __x | number of samples |
|------------------------------|---------|----------|--------|-------------------|
| | [final] | 1x | 4.1 | 5.0 |
| Incomplete buffer (10x) | 1x | 2.5 | 10.25 | 51.25 |
| MgCl ₂ | | 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 |
| H ₂ O | | 14.1 | 57.81 | 289.05 |
| MM volume (-template) | | 22 | 87.125 | 435.625 |
| DNA Template (from Clean-up) | | 3 | | |
| Final volume: | | 25 | | |

Plate Layout

| 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 |

- 1) Prepare 2 MM (Bacteria, Fungi) with forward primers, distribute 85 μl of MM to raw A (Bacteria) and E (Fungi) of a PCR plate
- 2) Add 3 μl Reverse primers with barcodes individually in raw A and E (4 barcoded reverse primers for bacteria, 4 barcoded reverse primers for fungi)
- 3) Add 3 μl of the digested control product to raw D and H
- 4) Distribute 22 μl 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

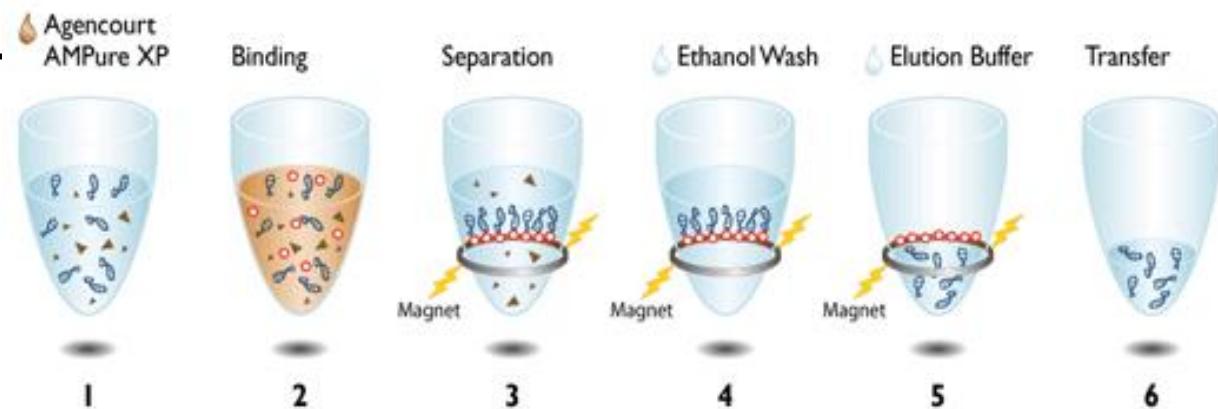
PCR program thermocycler

| Step | temp ° | time | |
|------|--------|------|------|
| 1 | 94° | 2' | |
| 2 | 94° | 30" | |
| 3 | 55° | 30" | 10 x |
| 4 | 72° | 60" | |
| 5 | 72° | 10' | |
| 6 | 4° | p | |

Day 3 – Tuesday March 28th

Purification – AMPure

- 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).



Day 4 – Wednesday March 29th

08:30 - 08:45

Briefing SR 1

08:45 - 09:00

Troubleshooting SR 1

Morning session: preparation of sequencing library - part III lab

09:00 - 09:30

Short talk: Experimental design for natural community profiling SR 1

09:30 - 11:00

Hands-on: DNA concentration measurement

11:00 - 11:15

Coffee break C

11:15 - 13:00

Hands-on: Pooling for library

13:00 - 14:00

Lunch C

Afternoon session: Amplicon sequencing using the Illumina MiSeq platform lab

14:00 - 14:30

Sequencing technologies for community profiling

14:30 - 16:00

Hands-on: Library preparation and sequencing using the MiSeq platform

16:00 - 16:30

Coffee break C

16:30 - 19:00

Poster session T

19:00 - 20:00

Dinner C

20:00 - 21:00

Evening lecture "*Experimental adaptation of microbial communities*" - **Stijn Spaepen** LH

Day 4 – Wednesday March 29th

Experimental design for microbial community profiling

Day 4 – Wednesday March 29th

- Type of experiments (Field vs. Greenhouse vs. Lab)
- Field: multiple sites, successional seasons/repli-cates, large number of plants: at least 8 samples, bulk soil sampling, adequate control for phyllosphere?
- Greenhouse: multiple soil types, randomized block design, full biological replicates, at least 8 samples per condition, unplanted pots as bulk soils.
- Lab: Gnotobiotic plant systems: use different types of systems, design of microbial inputs (maximize diversity, detection at strain level). full biological replicates, at least 8 samples per condition

Open discussion

Introduction to the practical session

Preparation of sequencing library (part II)

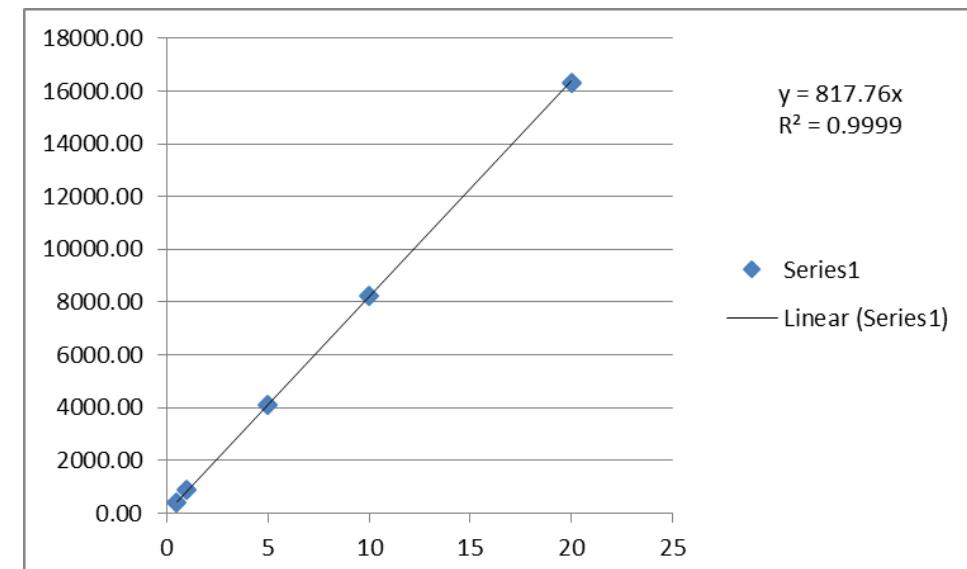
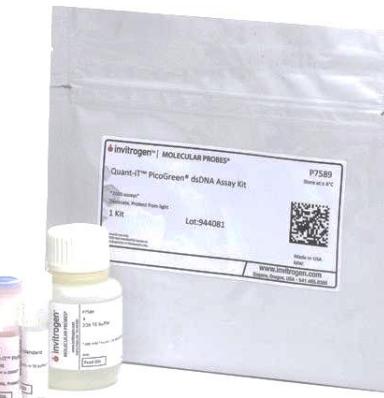
- DNA concentration using PicoGreen
- Pooling and purification of the library

Day 4 – Wednesday March 29th

Purification – Picogreen

- Prepare a dilution series (standard curve): 20 ng/ μ l, 10, 5, 1, 0.5, TE in 1x TE buffer.
- Dilute 200x picogreen in TE 1x (pico working solution).
- Add 40 μ l of pico working solution in wells of a qPCR plate.
- Add 4 μ l of sample or 4 μ l 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).
- Subtract TE fluorescence background.
- Plot standard curve.
- Divide sample fluorescence intensity by slope of standard curve.

Quant-iT PicoGreen dsDNA Assay Kit



Day 4 – Wednesday March 29th

Pooling and purification of library

Aim: equimolar pooling of all barcoded samples into one sequencing library

→ equimolar = same amount in ng per sample if products have the same size.

- Define the amount of pooled product (e.g. 30 ng) per amplicon
 - look at the highest concentrated sample – pipetted volume should not be lower than 1 µl.
- Calculate the required amount of each sample to be pooled.
 - = amount of pooled product / concentration sample (in ng)
- AMPure twice the pooled library (first elution: 200 µl / second elution: 80 µl).
- Measure the library concentration by PicoGreen or **Qubit assay**.
- Pool the libraries from individual marker loci together into one library in equimolar amounts.
- Measure the library concentration by PicoGreen or Qubit assay.
- Other QC: gel electrophoresis of library / Bioanalyzer or TapeStation (capillary/gel electrophoresis)

Day 3 – Tuesday March 28th

Sequencing technologies for community profiling

Day 4 – Wednesday March 29th

Introduction to Sequencing technologies for community profiling

Sanger sequencing (1977 – present)

Gel based

Capillary system

Next Generation sequencing (2005 – present)

454

Ion Torrent

Illumina

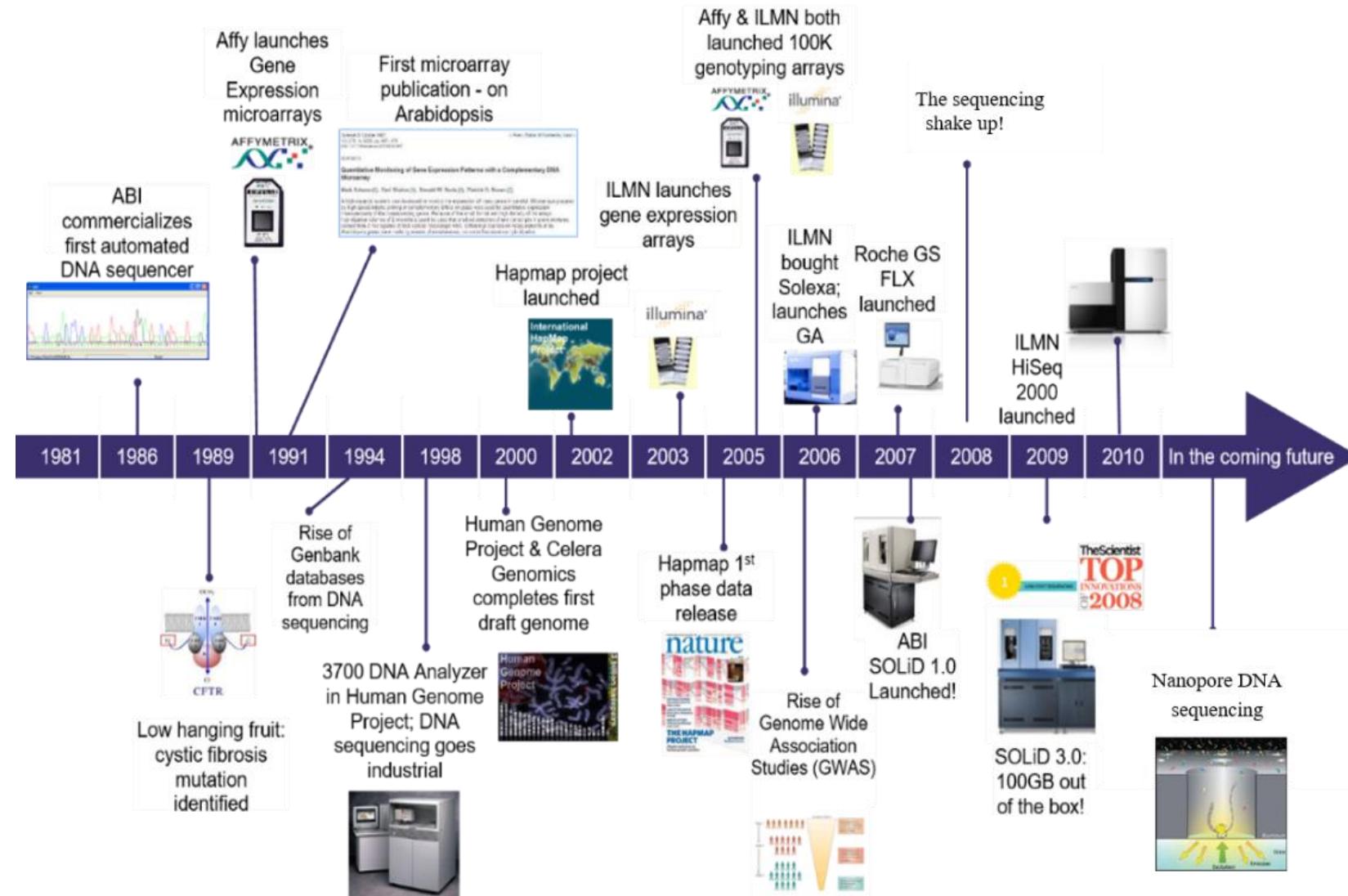
3rd generation sequencing (2012 – present)

PacBio or SMRT sequencing

Oxford Nanopore

Day 4 – Wednesday March 29th

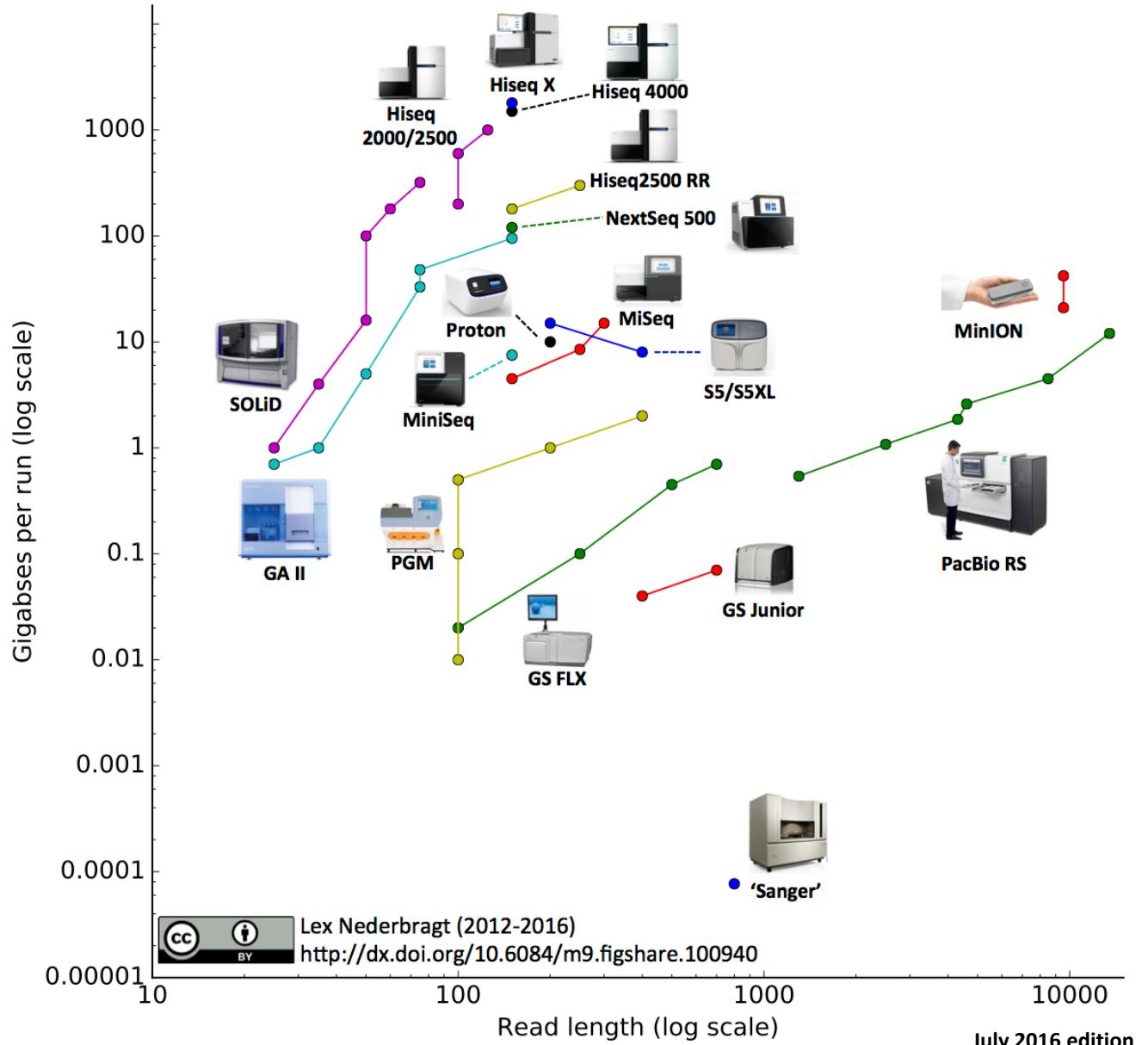
Developments in sequencing technologies



Day 4 – Wednesday March 29th

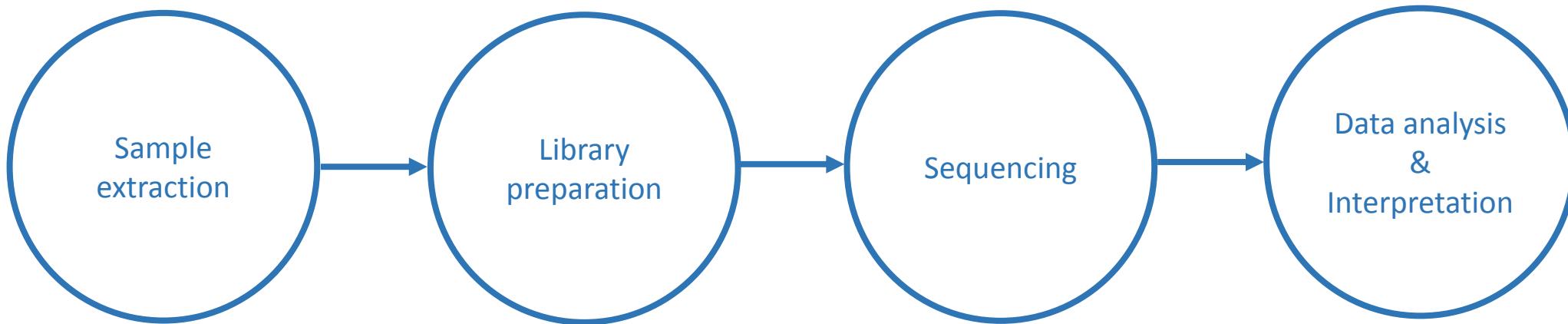
Developments in high throughput sequencing

Full run throughput in gigabases (billion bases) is plotted against single-end read length for the different sequencing platforms, both on a log scale.



Day 4 – Wednesday March 29th

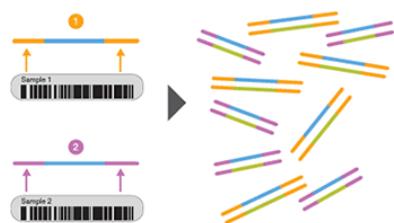
Universal NGS workflow for Illumina



Genomic DNA or RNA



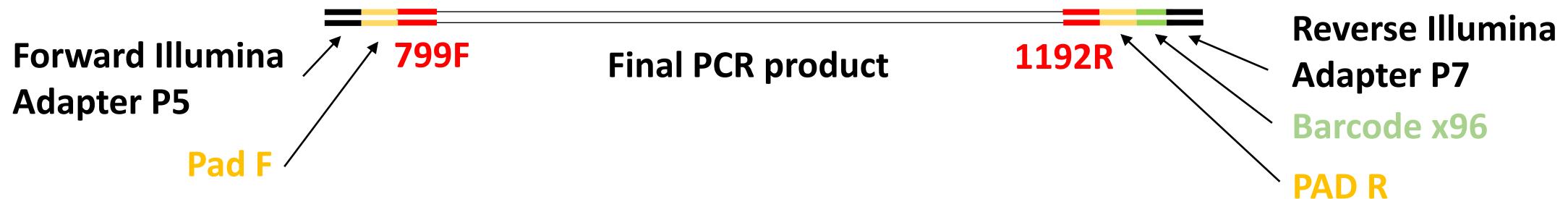
Bringing DNA/RNA into a format that is usable to the sequencer



On Illumina platform



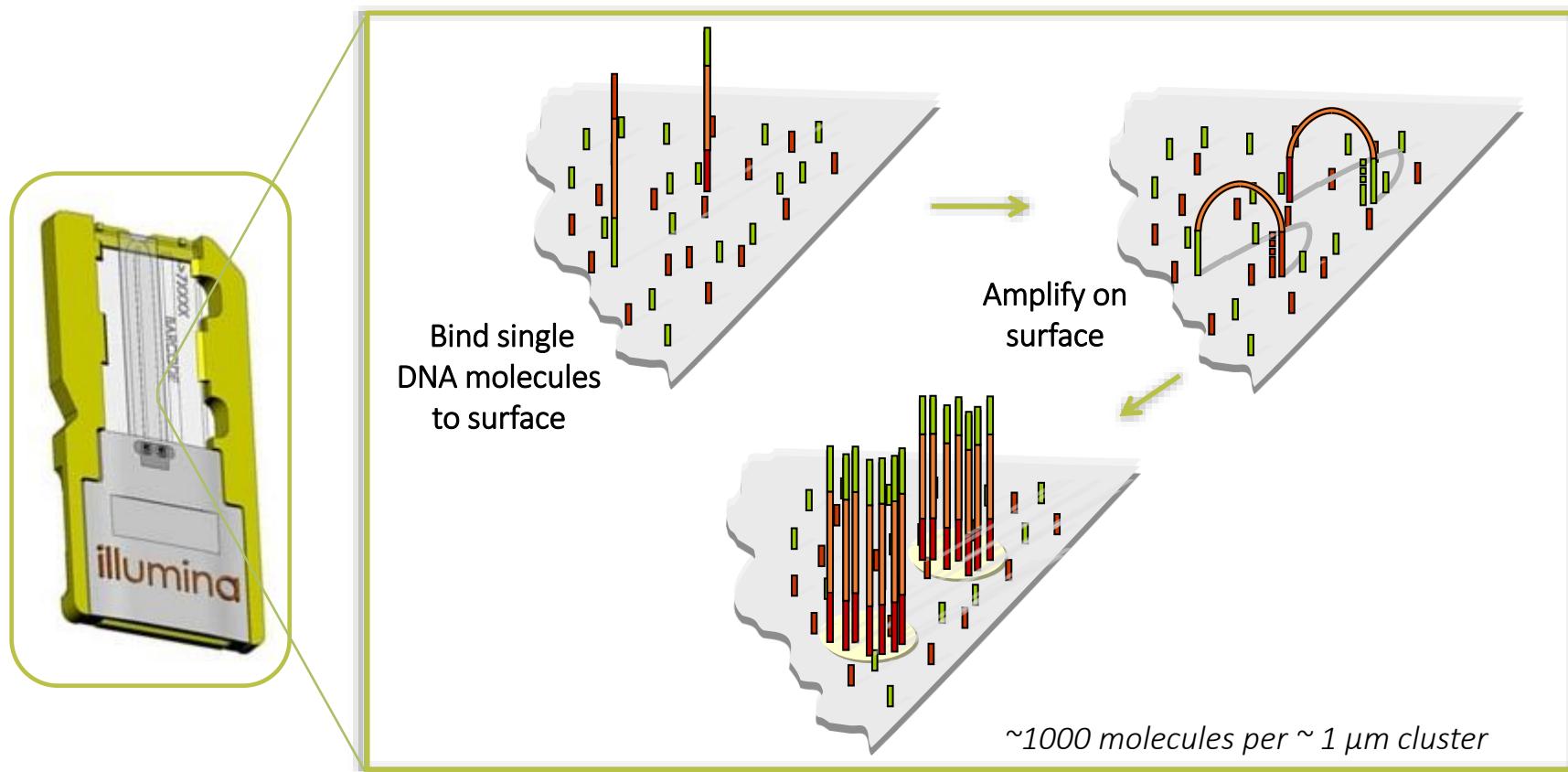
Day 4 – Wednesday March 29th



The aim of the sample prep step is to obtain nucleic acid fragments with adapters (and barcodes) attached on both ends

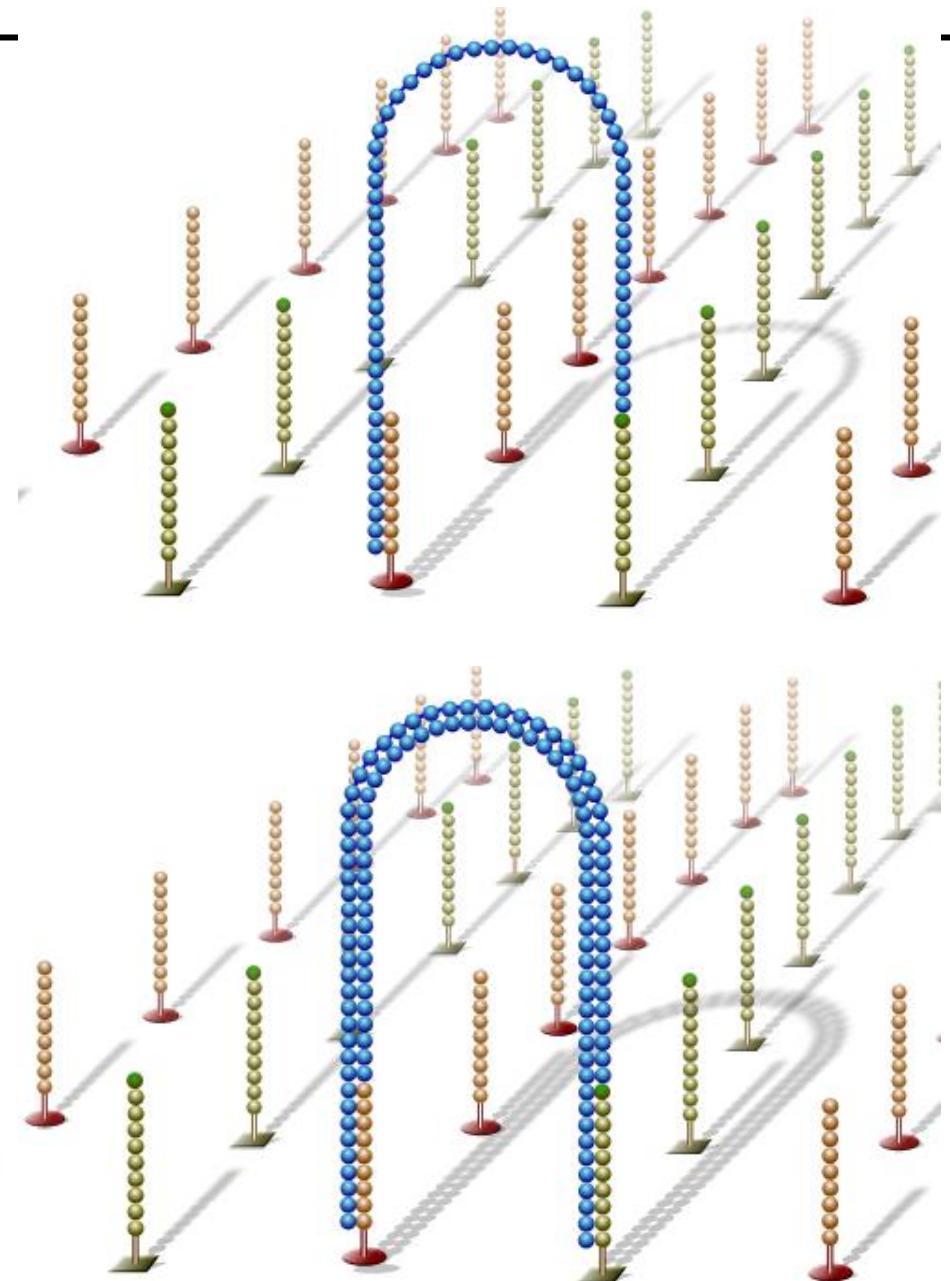
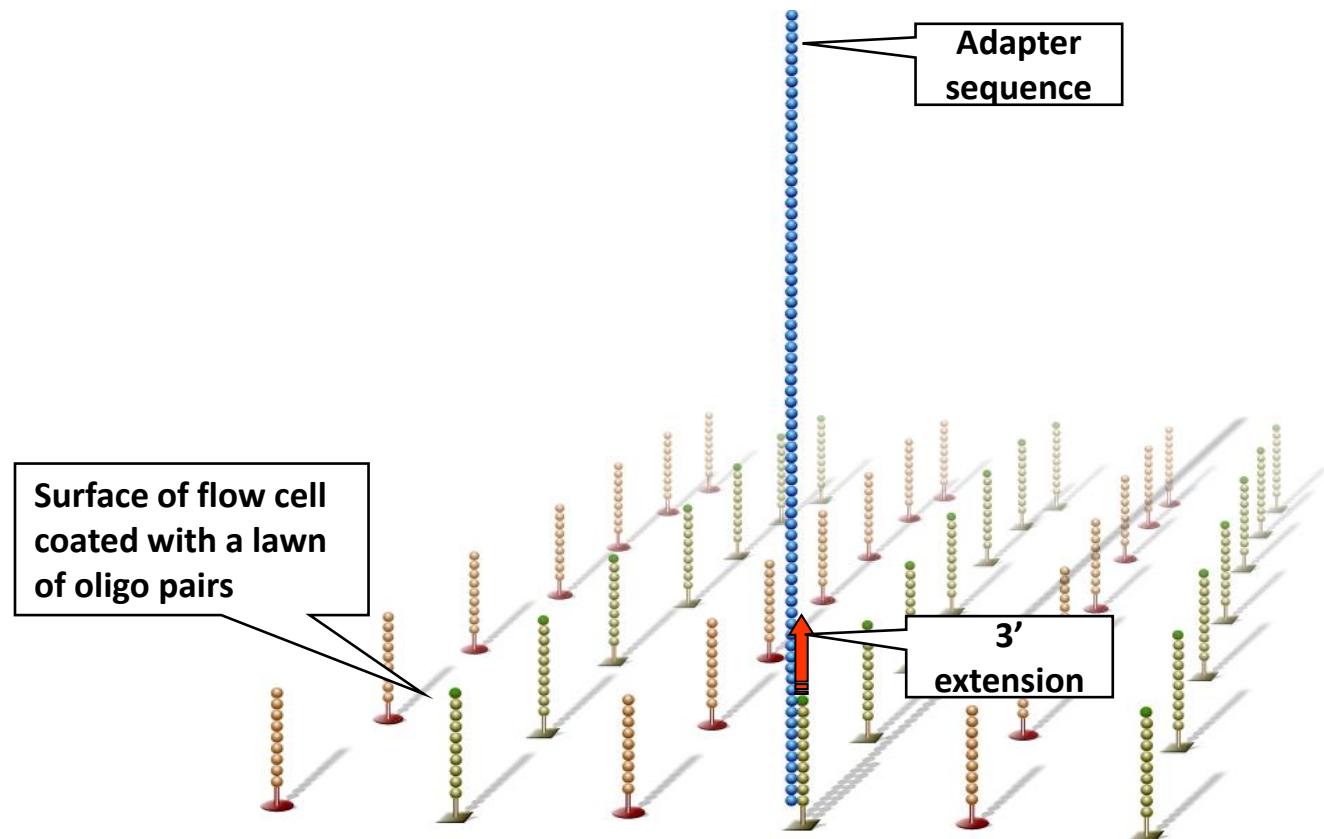
Day 4 – Wednesday March 29th

Cluster Generation Overview

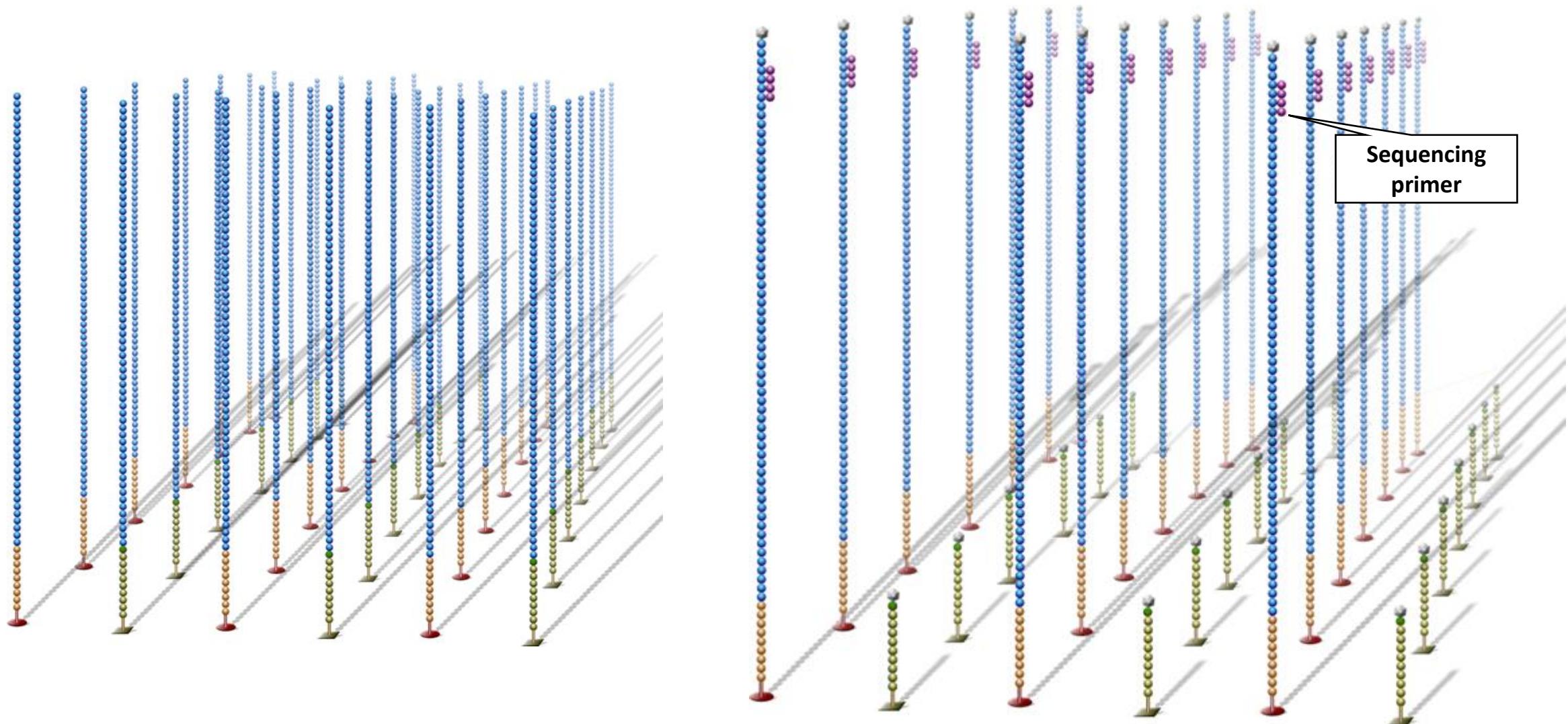


Day 4 – Wednesday March 29th

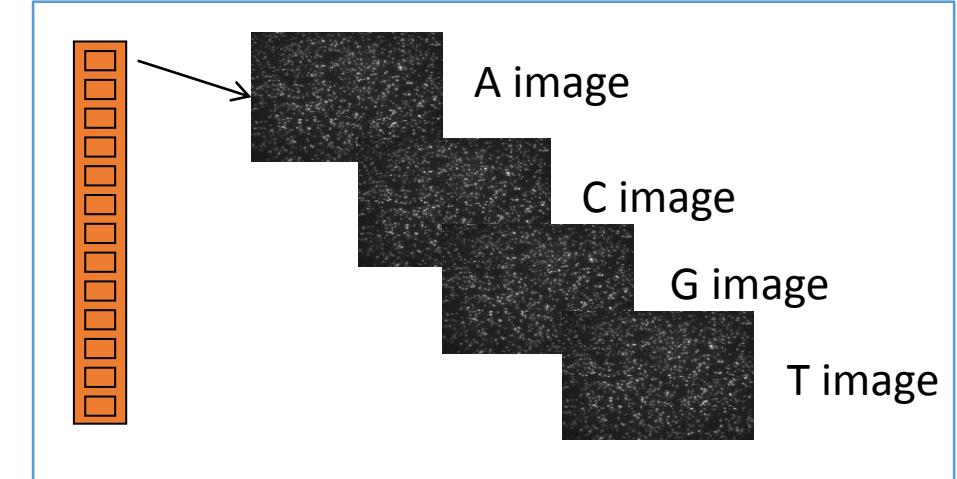
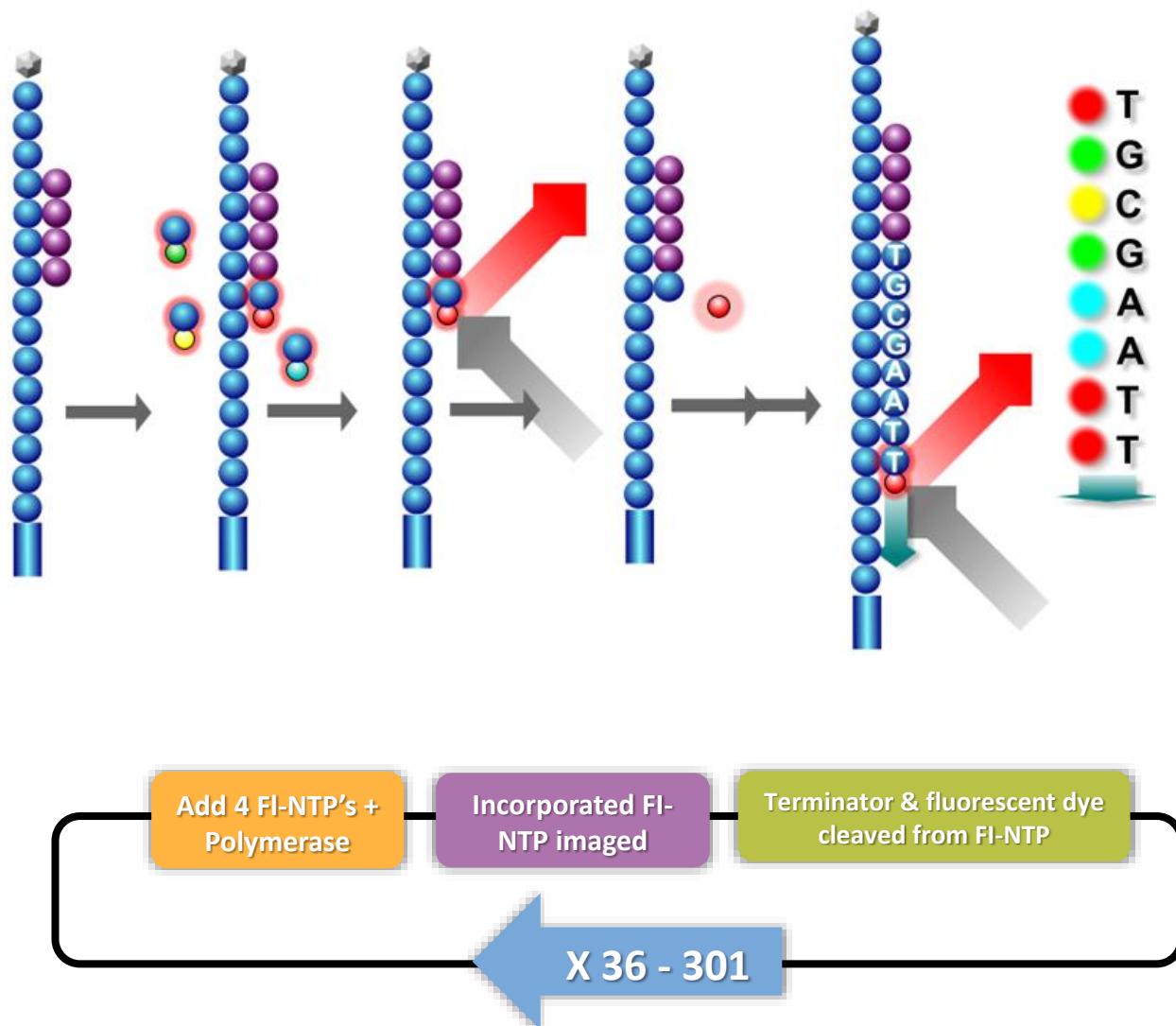
Amplification of bound molecules to cluster of molecules by 3' synthesis and bridge formation.



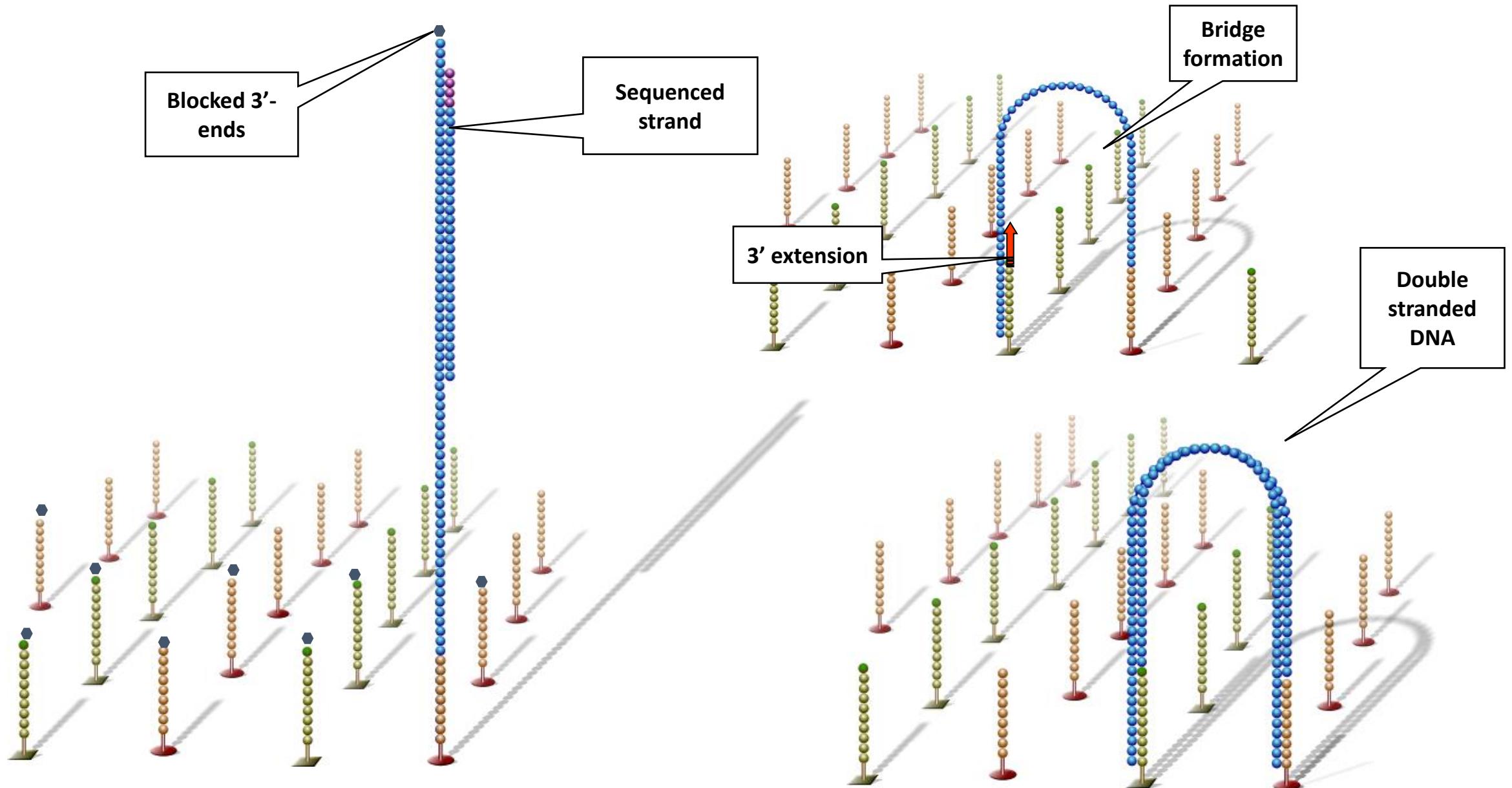
Day 4 – Wednesday March 29th



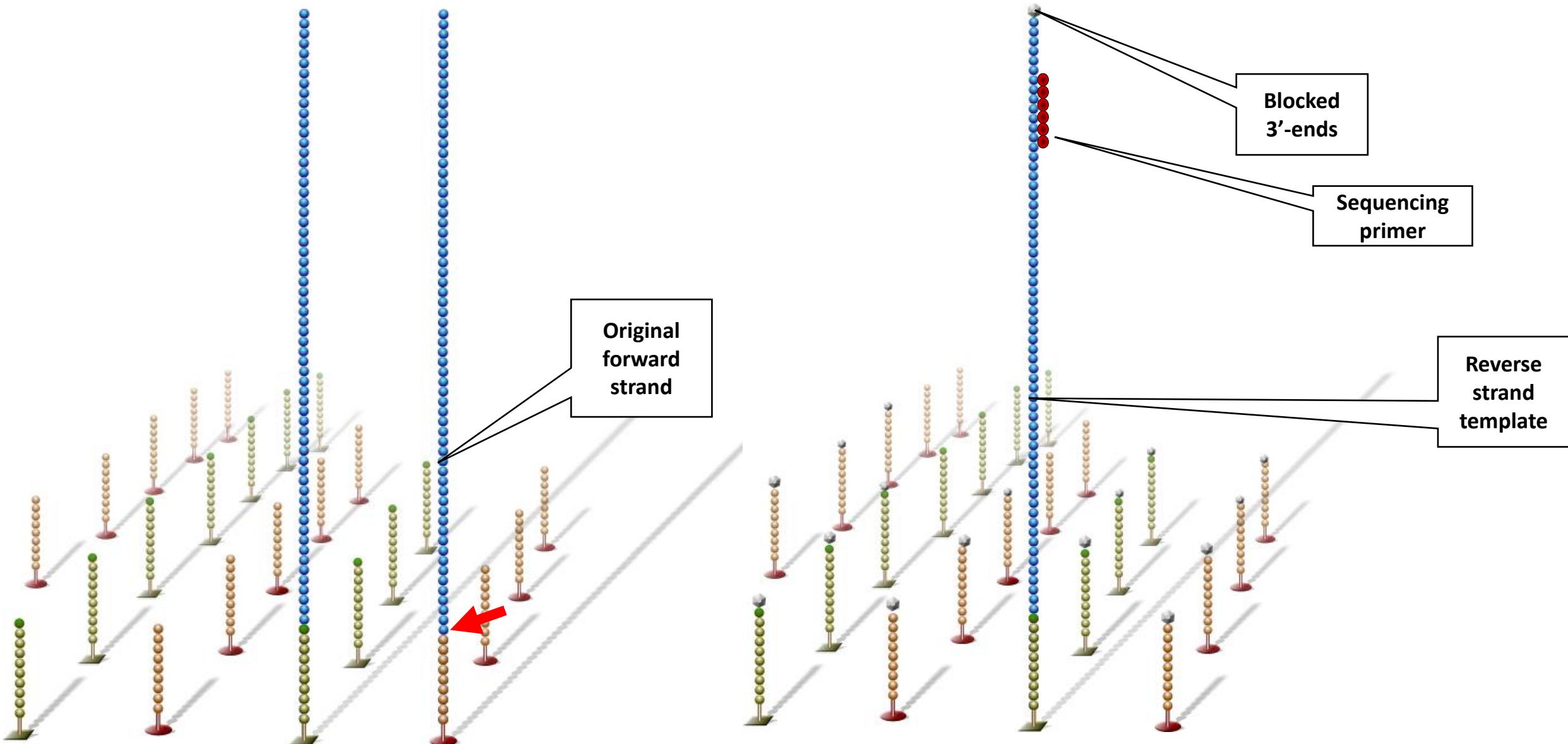
Day 4 – Wednesday March 29th



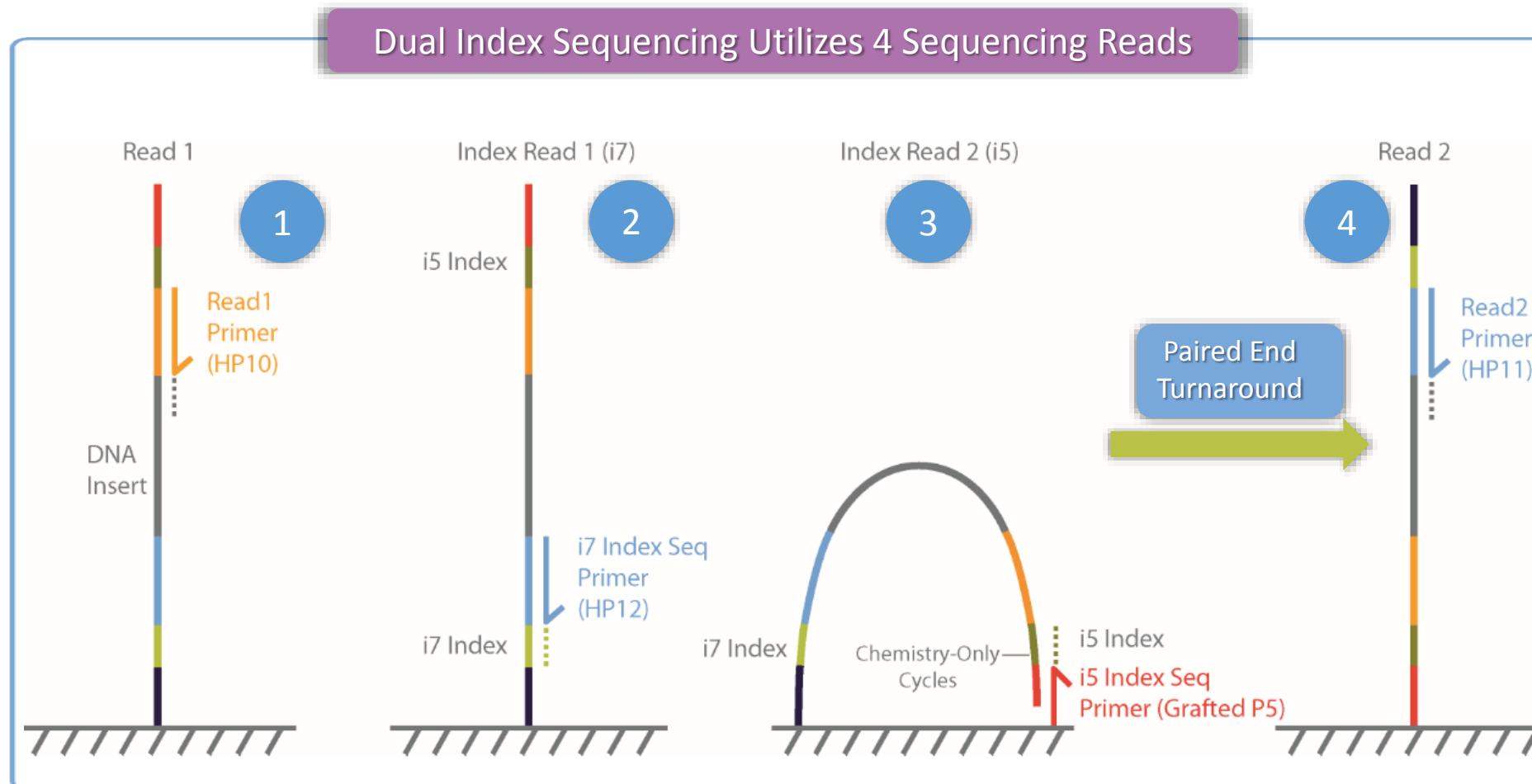
Day 4 – Wednesday March 29th



Day 4 – Wednesday March 29th



Day 4 – Wednesday March 29th



Day 4 – Wednesday March 29th

Diverse Libraries

vs

Low Diversity Libraries

AACGAGACGCATTC
TTCTGACGAGTAAC
ACACAGGGCTACTAC
GCGATAACCATGATG

ACATAGGCTACTAC
ACATAGCCTACTAC
ACATAGGCTACTAC
ACATAGGCTACTAC

Critical calculations that occur in the first 11 cycles:

- Cluster identification
- Matrix calculation
- Image registration
- Focusing, on some sequencers

Low diversity or mono template can interfere with some of these calculations

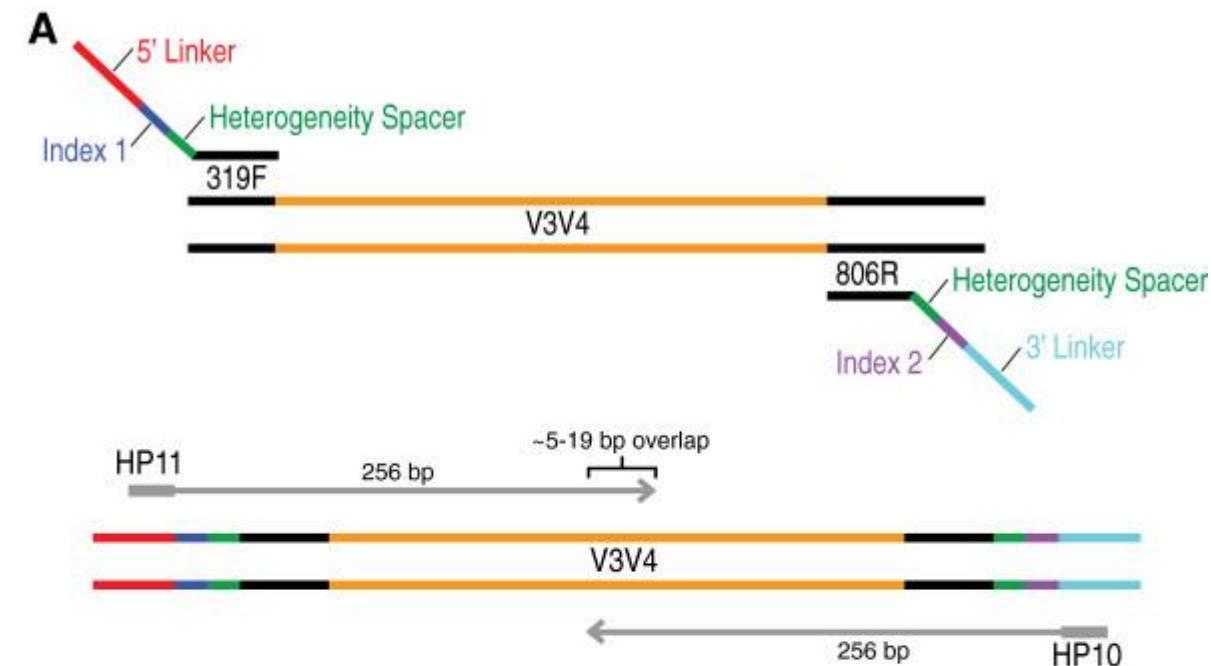
Day 4 – Wednesday March 29th

Low Diversity Libraries

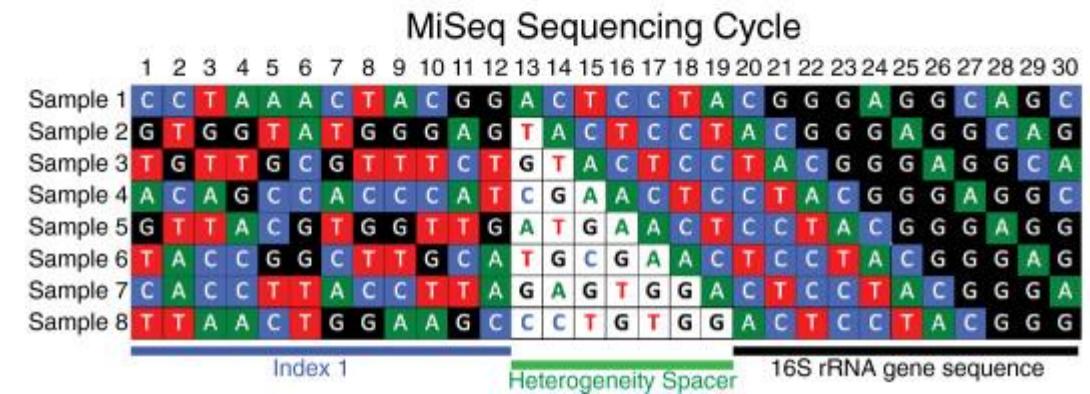
ACATAGGCTACTAC
ACATAGCCTACTAC
ACATAGGCTACTAC
ACATAGGCTACTAC

How to overcome sequencing problems related to low-diversity libraries?

- Keep cluster density low (max 800k cluster /mm²)
- Spike in PhiX control (at least 5%)
- Use out-of-frame primers with heterogeneity spacer in order to have not the same base at each position



B



Day 5 – Thursday March 30th

08:30-08:45

Briefing **SR 1**

08:45-09:00

Troubleshooting **SR 1**

Opening session: Computational methods for microbial ecology

09:00 - 10:00

Lecture "*Computational methods for microbial ecology*" - **Ruben Garrido-Oter** **LH**

Morning session: from raw reads to OTU tables **SR 1**

10:00 - 10:15

Coffee break **SR 1**

10:15 - 10:45

Short talk: State-of-the-art approaches for amplicon data analysis

10:45 - 13:00

Hands-on: Read quality control, sample demultiplexing and OTU clustering

13:00 - 14:00

Lunch **C**

Afternoon session: Taxonomic characterization and diversity analyses **SR 1**

14:00 - 14:30

Short talk: Taxonomic characterization and diversity analyses

14:30 - 16:00

Hands-on: Taxonomic classification and indices of alpha-and beta-diversity

16:00 - 16:30

Coffee break **SR 1**

16:30 - 18:00

Troubleshooting

19:00 - 21:00

Dinner **C**

Day 6 – Friday March 31st

08:30 - 08:45

Briefing SR 1

08:45 - 09:00

Troubleshooting SR 1

Opening session: Mechanisms underlying interactions between arbuscular mycorrhizal fungi and plants

09:00 - 10:00

Lecture "*The interaction of plants and their largest endosymbionts, arbuscular mycorrhizal fungi*" - **Maria Harrison** LH

Morning session: Diversity analyses and community data visualization in R SR 1

10:00 - 11:00

Hands-on: Alpha- and beta-diversity

11:00 - 11:15

Coffee break SR 1

11:15 - 13:00

Hands-on: Multidimensional scaling and ordination methods
(PCoA, MDS, etc.)

13:00 - 14:00

Lunch C

Afternoon session: Multivariate statistical techniques in plant microbiota studies SR 1

14:00 - 15:00

Data visualization

15:00 - 16:00

Hands-on: Data normalization and OTU enrichment tests

16:00 - 16:15

Coffee break SR 1

16:15 - 16:45

Short talk: Statistical models to assess the effect of environmental variables

16:45 - 18:00

Hands-on: Constrained ordination methods (CPCoA, etc.)

19:00 - 20:00

Dinner C

Keynote session: Multi-Omics of Soil Microbiomes

20:00 - 21:00

Keynote lecture "*Multi-Omics of Soil Microbiomes*" - **Janet Jansson** LH

Day 7 – Saturday April 1st

Shiji and Jack

| | | |
|---------------|--------------------------|--|
| 10:00 - 10:45 | Round table | SR 1 |
| 10:45 - 11:00 | Coffee break | SR 1 |
| 11:00 - 12:30 | Poster session | T |
| 12:30 -... | Transfer to city center: | Cultural social event in Cologne city center |

Day 9 – Monday, April 3rd

08:30 - 08:45

Briefing SR 1

08:45 - 09:00

Troubleshooting SR 1

Keynote session: Functions of the plant microbiota LH

09:00 - 10:00

Keynote lecture "*Culture collections and synthetic communities as tools to understand microbial community functions*" - **Paul Schulze-Lefert**

10:00 - 11:00

Lecture "*Induced systemic resistance in a community context*" - **Corné Pieterse**

11:00 - 11:15

Coffee break SR 1

Morning session: Building bacterial and fungal culture collections - part I lab

11:15 - 11:45

Introduction

11:45 - 13:00

Hands-on: Preparation of materials

13:00 - 14:00

Lunch C

Afternoon session: Building bacterial and fungal culture collections - part II lab

14:00 - 16:00

Hands-on: Isolation of bacterial and fungal isolates from plant tissues using traditional plating methods

16:00 - 16:15

Coffee break C

16:15 - 18:30

High-throughput establishment of bacterial culture collections using the limited dilution method

18:30 - 19:00

Closing discussion

19:00 - 21:00

Dinner C

Introduction to the practical session

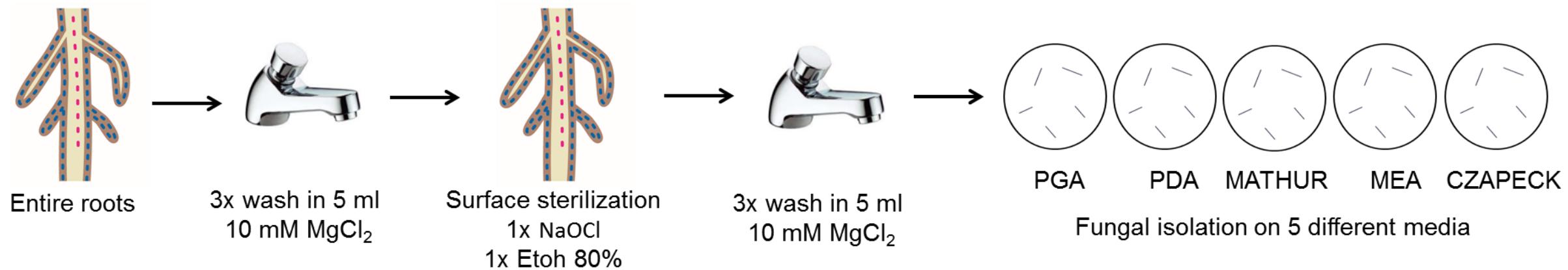
Stéphane/Rafal

Isolation of bacterial and fungal isolates from plant tissues using traditional plating methods

High-throughput establishment of bacterial culture collections using the limited dilution method

Day 9 – Monday, April 3rd

Isolation of fungal isolates from plant tissues using traditional plating methods



Fungal isolation from roots

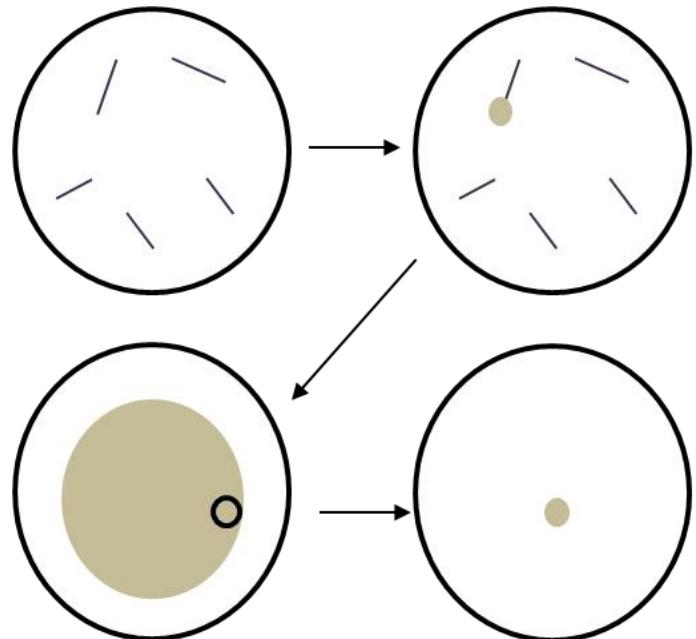
1. Clean roots (remove soil as much as possible)
2. Wash roots 3 times in sterile water or 10 mM MgCl₂ (5 min each). Rotate the 12 mL falcon tubes.
3. Wash roots in 80% EtOH (1 min). Rotate the 12 mL falcon tubes
4. Wash roots in 3% NaClO (Sodium hypochlorite) in water (1min). Rotate the 12 mL falcon tubes
5. Wash roots 3X in water or 10 mM MgCl₂ (5min). Rotate the 12 mL falcon tubes
6. Transfer roots in petri dishes and cut the roots in 0.5 cm pieces
7. Put pieces on different agar media without antibiotics (Mm-, PGA/PDA-, MEA- and CDA-)
8. After 2-3 days start transferring fungi to PDA plates supplemented with antibiotics

Day 9 – Monday, April 3rd

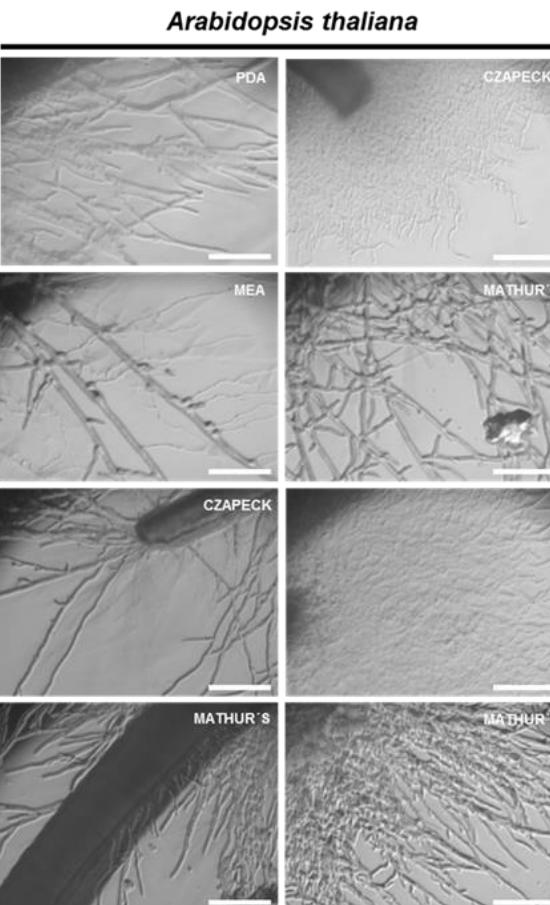
Isolation of fungal isolates from plant tissues using traditional plating methods

Surface sterilized root fragments

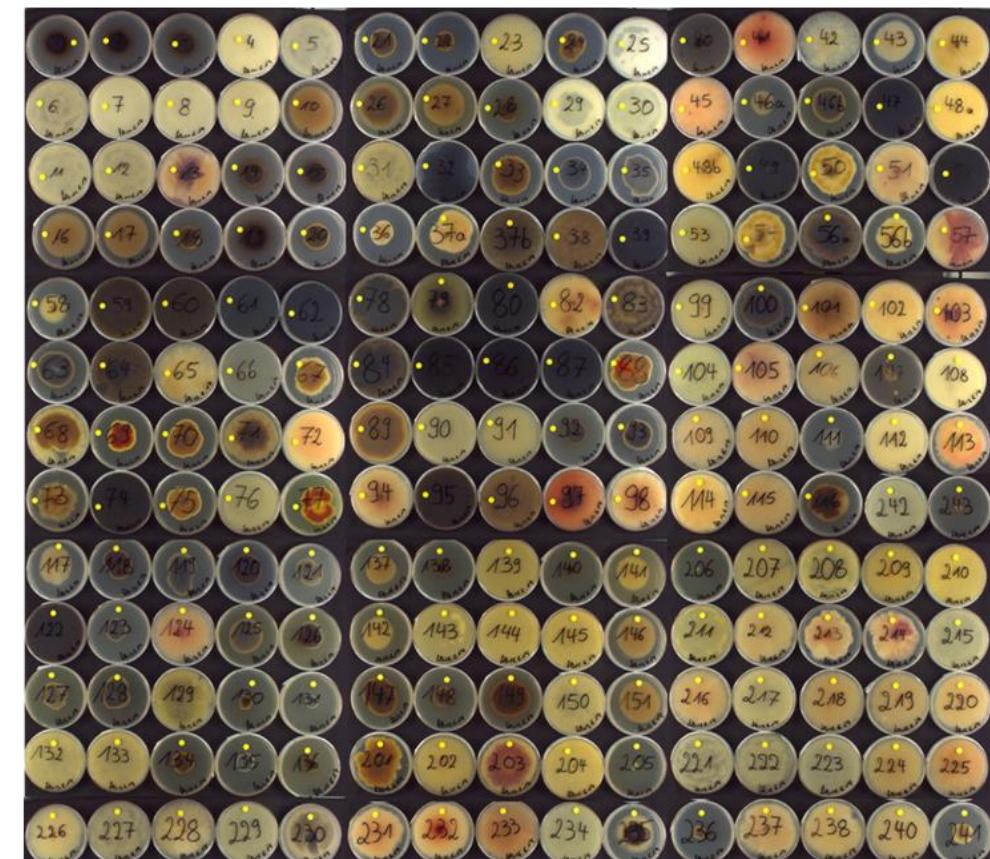
Fungal mycelium emerging



Transfert in PDA
without antibiotics

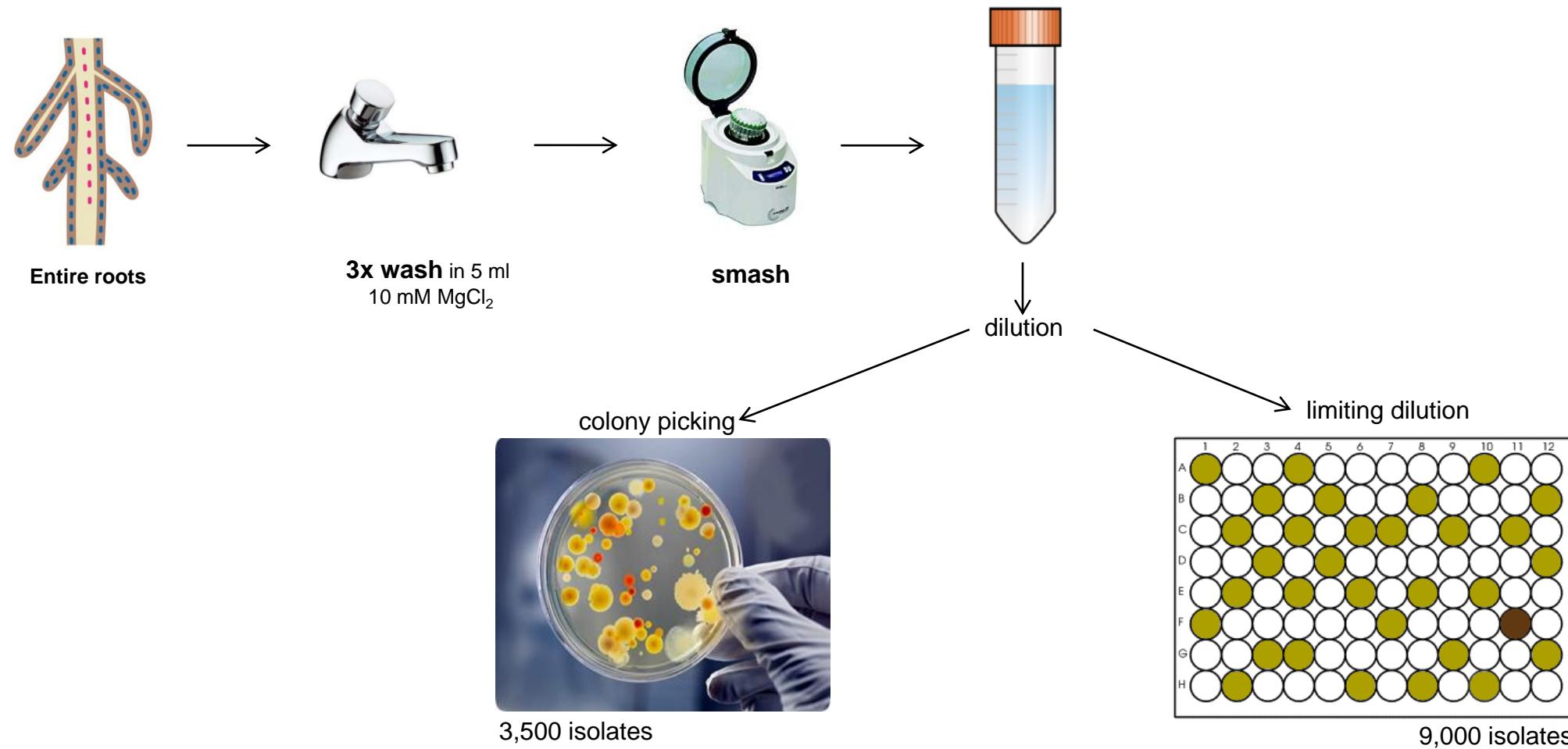


Fungal reference culture collection



Day 9 – Monday, April 3rd

Isolation of bacterial isolates from plant tissues using traditional plating methods



Day 9 – Monday, April 3rd

Building the bacterial culture collections

Materials

(limiting dilution method)

- 10 mM MgSO₄ (45 ml per sample)
- 10% TSB (Tryptic Soy Broth, 250ml per sample)
- Scalpel and forceps
- Metal beads $\phi = 1\text{-}3 \text{ mm}$ (3 per sample)
- 50 ml tubes (11 per sample)
- Screw-cap 2ml tubes
- Tissuelyser/Precellys device
- 96-well plates
- Multichannel pipet (100-200 μl)
- Multichannel pipet reservoir

(only for traditional plating method)

- TSA plate (50%)
- Sterile glass beads 3 mm

Day 9 – Monday, April 3rd

Building the bacterial culture collections

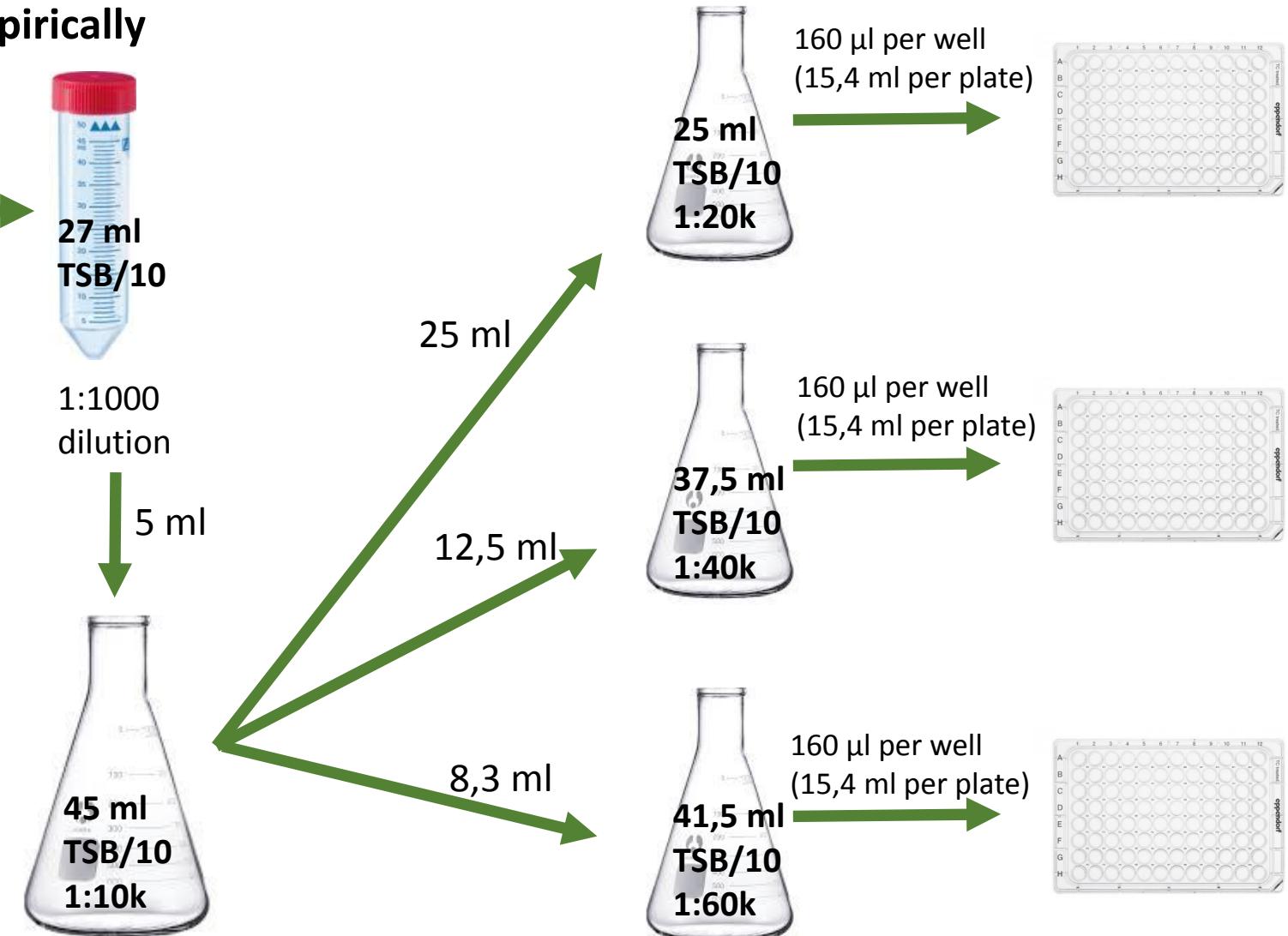
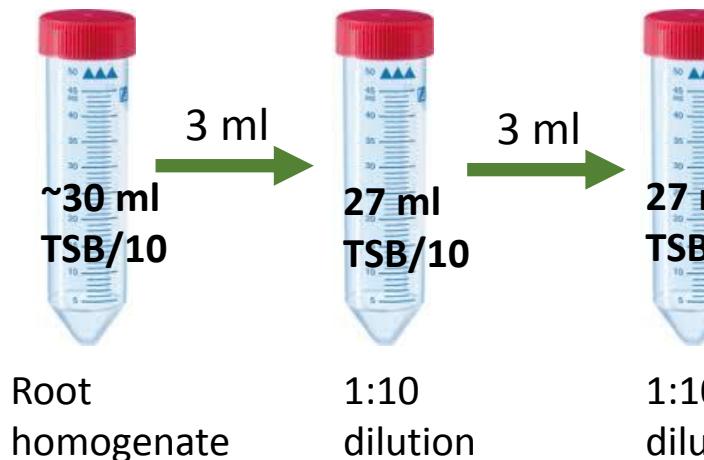
Protocol

1. Extract plants from the pot, remove excessive amounts of soil attached to the root
2. For following steps, use 4 cm-long root fragments collected 1 cm down from the rosette
3. Transfer root fragments to 50 ml tubes containing 15 ml 10 mM MgSO₄, wash on rotary shaker at 80 rpm for 10 to 20 min
4. Repeat washing 3 times in total, each time in new 50 ml tube containing 15 ml 10 mM MgSO₄
5. Transfer root fragments to 2 ml tubes containing 500 µl 10% TSB medium and 3 metal beads, homogenize on Precellys for 30 sec at 5000 rpm
6. Combine root slurry with 30 ml 10% TSB medium in 50 ml falcon tube, mix and wait 15-30 min for plant material to sediment
7. In 50 ml falcon tubes, prepare the intermediate dilutions of root homogenate: 1:10 to 1:10k, as well as plating dilutions: 1:20k, 1:40k, 1:60k. Avoid uptake of the plant material. Use 10% TSB.
8. Inoculate 3 96-well plates with each of the dilutions of root homogenate: 1:20k, 1:40k, 1:60k. Use 160 µl per well (15,4 ml per plate)
9. Seal the plates and incubate for 15-20 days at RT without shaking

Day 9 – Monday, April 3rd

Limiting dilution method – if 70% of the wells are empty, (statistically) 95% of the remaining ones contain a single bacterial isolate

The correct dilution has to be verified empirically



Important parameters:

- Host species
- Soil type
- Individual extraction rounds

Day 9 – Monday, April 3rd

Two-step barcoding applied for culture collection preparation

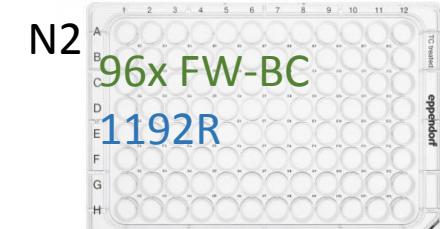
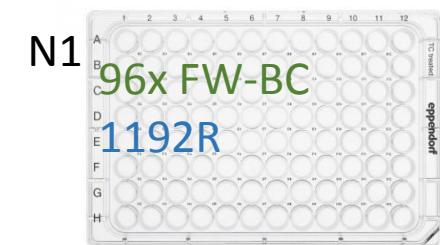
Step 1: well barcoding

Barcoded forward primer (FW-BC)

AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXXXXXXX**GACTGCGACTGGCGAACMGGATTAGATA**CCKG

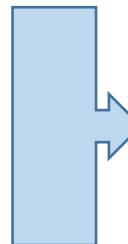
Reverse primer (1192R)

*ACGT*CATCCCCACCTTCC



POOLING EACH PLATE IN ONE WELL

Step 2: plate barcoding



POOLING AND
SEQUENCING

Forward primer
AATGATACGGCGACCAC

Barcoded reverse primer (RV-BC)

*CAAGCAGAAGACGGCATACGAGATXXXXXX*XXXXXXXXX**CAGCCATTAGTGT**CACGTCCCCACCTTCC

Bold: Illumina Adapter

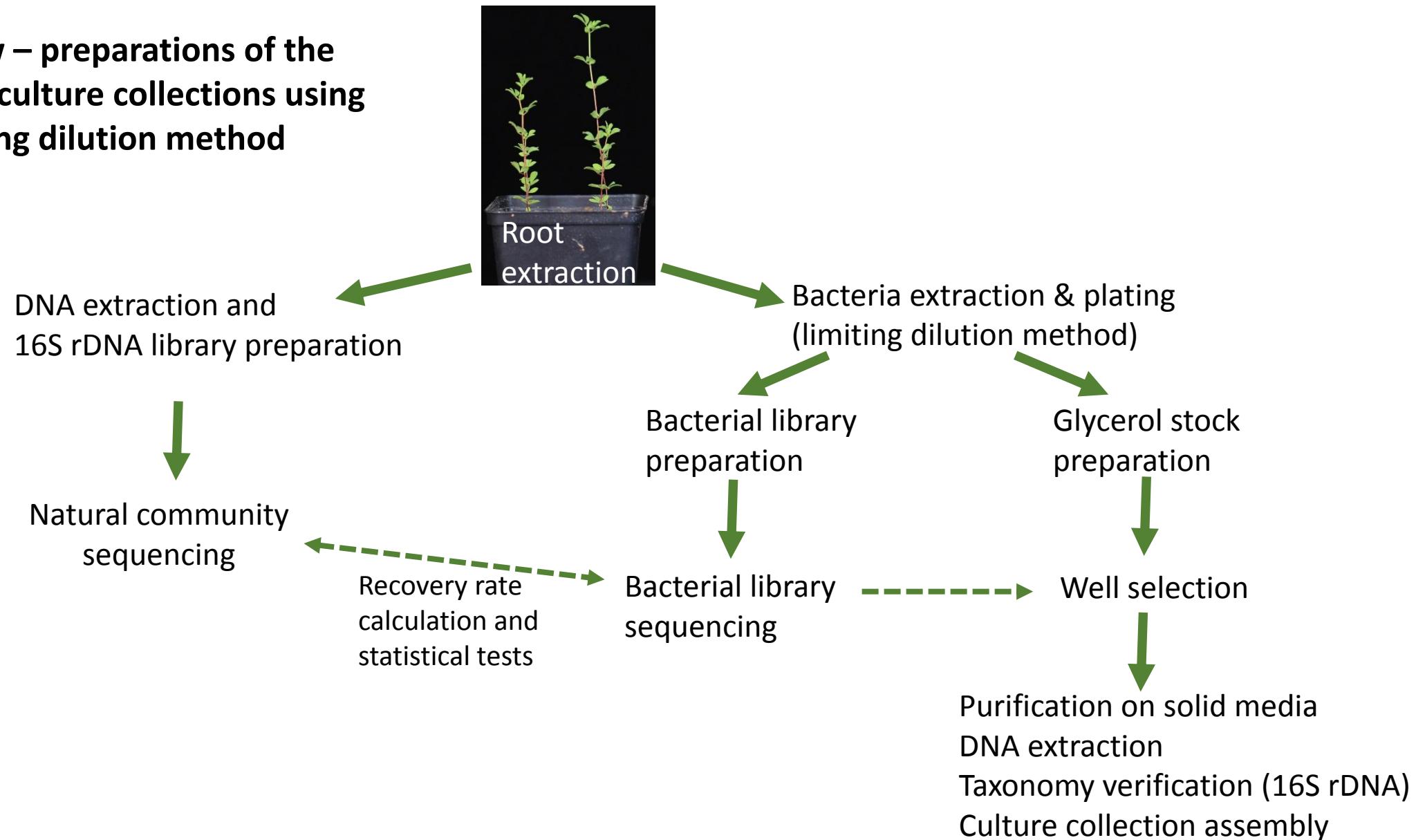
XXXX: Barcode

Underscore: PADs

Italics: 799F or 1192R primer

Day 9 – Monday, April 3rd

Workflow – preparations of the bacterial culture collections using the limiting dilution method



Day 10 – Tuesday, April 3rd

08:30 - 08:45

Briefing SR 1

08:45 - 09:00

Troubleshooting SR 1

**Morning session: Synthetic microbial communities (SynComs) and gnotobiotic systems
- part I lab**

09:00 - 09:30

Short talk: Description of gnotobiotic systems

09:30 - 10:30

Hands-on: Preparation of 'Calcined Clay' systems

10:30 - 10:45

Coffee break C

10:45 - 13:00

Hands-on: Preparation of 'FlowPot' systems

13:00 - 14:00

Lunch C

Afternoon session: SynComs and gnotobiotic systems - part II lab

14:00 - 16:00

Hands-on: Assembling complex microbial consortia

16:00 - 16:15

Coffee break C

16:15 - 18:30

Inoculation of gnotobiotic systems and sowing seeds

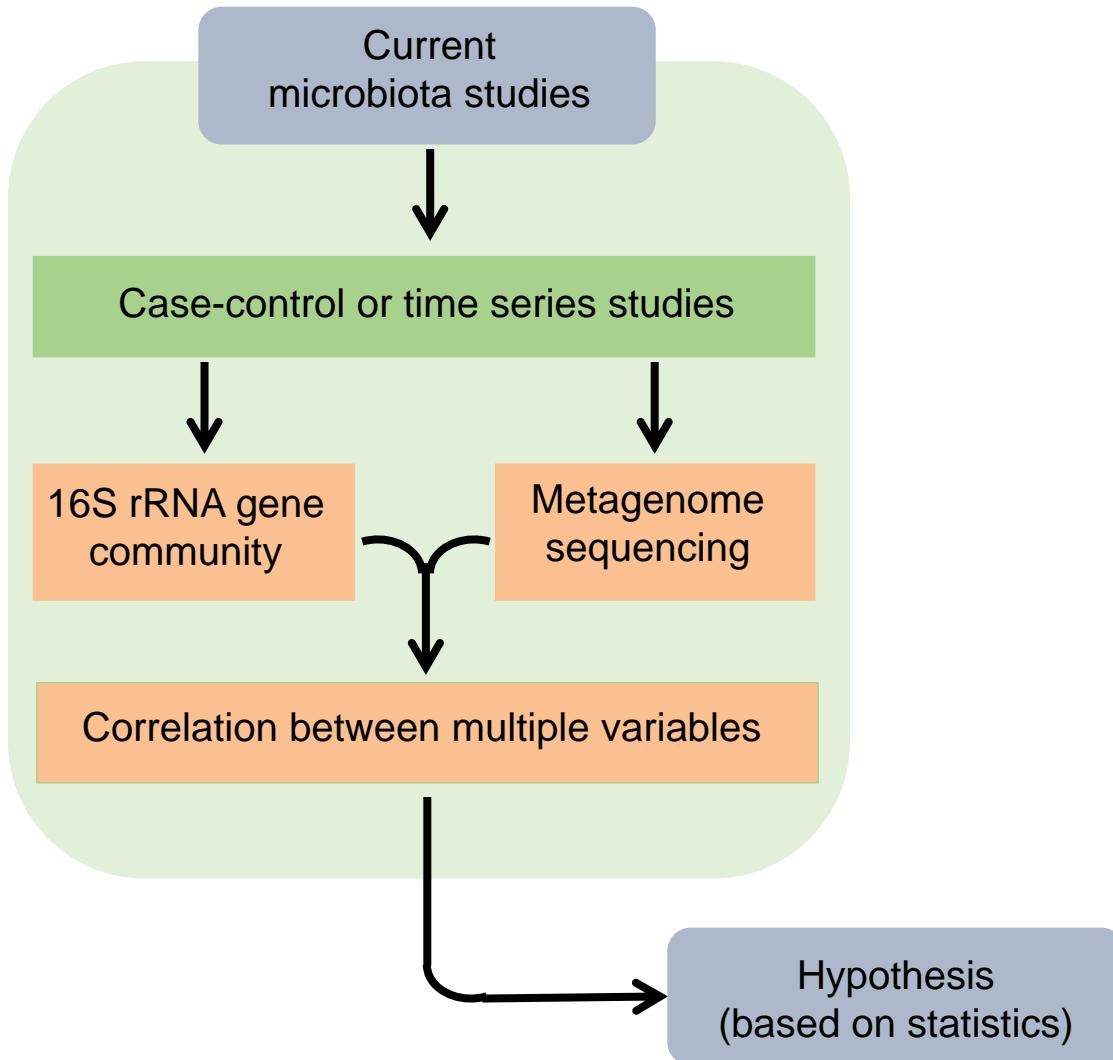
18:30 - 19:00

Closing discussion

19:00 - 21:00

Dinner C

Day 10 – Tuesday, April 3rd



Day 10 – Tuesday, April 3rd



Pros

- **Most reduced/simple system**
- **Cheap**
- **High throughput**
- **No prone to external contamination**
- **Defined nutrient conditions**
- **Monitoring root growth**

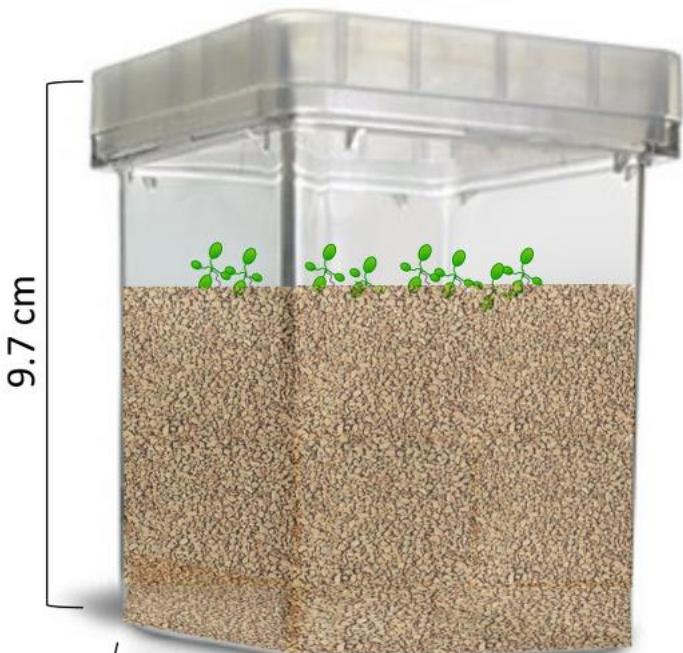


Cons

- **Most artificial/ furthest away from natural conditions**
- **No root/leaf compartmentalization**
- **No matrix effect**
- **Short term-experiments (3 weeks)**
- **Problems with high diversity inputs**

Day 10 – Tuesday, April 3rd

'Calcined-clay' system



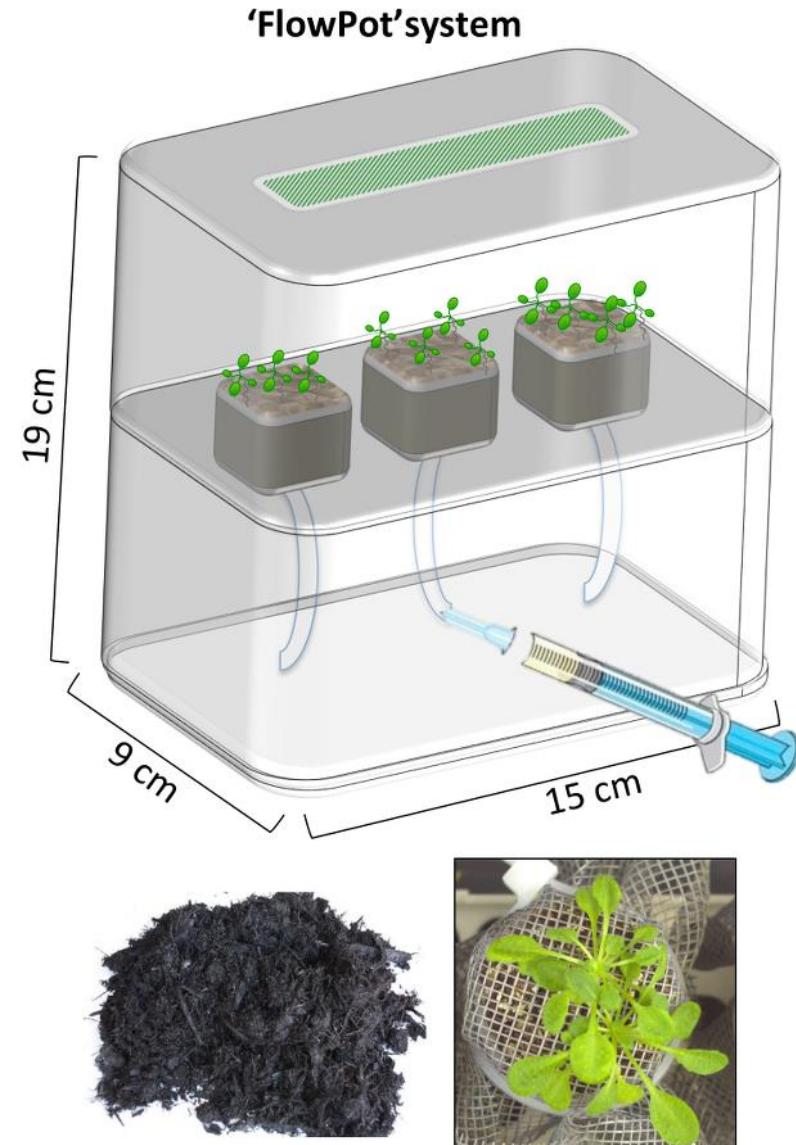
Pros

- High throughput
- No prone to external contamination
- Defined nutrient conditions (with exceptions)
- Root/leaf compartmentalization
- Long-term experiments
- Inoculation of complex bacterial SynComs (fungi ?)

Cons

- Artificial (only clay particles)
- Low survival in matrix due to low C content
- High humidity (aeration ?)
- Slow plant growth (low turnover)

Day 10 – Tuesday, April 3rd



Pros

- Mimicking natural soil
- High carbon content (high survival of microbes)
- Adapted for highly complex microbial consortia
- Root/leaf compartmentalization
- Adaptable for use with natural soil

Cons

- Low throughput
- Prone to external contamination
- Cannot apply nutritional stress
- Expensive and time consuming set-up
- Laborious harvesting process

Day 11 – Wednesday, April 4th

08:30 - 08:45

Briefing SR 1

08:45 - 09:00

Troubleshooting SR 1

Morning session: Bioinformatic analysis of sequence-indexed culture collections SR 1

09:00 - 10:30

Hands-on: Processing of IRL data and estimation of recovery rates

10:30 - 10:45

Coffee break SR 1

10:45 - 12:00

Hands-on: Identification of isolates in culture collections

12:00 - 13:00

Troubleshooting

13:00 - 14:00

Lunch C

Afternoon session: Analysis of SynComs data SR 1

14:00 - 16:00

Hands-on: Reference-based methods for community analysis

16:00 - 16:15

Coffee break SR 1

16:15 - 17:00

Short talk: Computational and statistical analyses of SynComs

18:00 - 21:00

Barbeque T

Day 12 – Thursday, April 5th

08:30 - 08:45

Briefing **SR 1**

08:45 - 09:00

Troubleshooting **SR 1**

Morning session: Whole-genome comparative analysis of microbial isolates **SR 1**

09:00 - 09:15

Introduction

09:15 - 09:45

Short talk: Overview of bioinformatics tools for whole-genome sequence analysis

09:45 - 10:45

Hands-on: Assembly, gene prediction and functional annotation

10:45 - 11:00

Coffee break **SR 1**

11:00 - 13:00

Hands-on: Phylogenetic methods for the analysis of whole genome data

13:00 - 14:00

Lunch **C**

Afternoon session: Metagenome and metatranscriptome approaches for microbiota studies **LH**

14:00 - 15:00

Lecture "*Insights from benchmarking computational methods for shotgun metagenomics*" - **Alice McHardy**

Closing session: Perspectives on plant-associated microbiota **LH**

15:00 - 16:00

Lecture "Sequence-based approaches to plant microbiomes" - **Susannah Tringe**

16:00 - 16:30

Coffee break **SR 1**

16:30 - 18:00

Open topic discussion with suggestions from participants

18:00 - 19:00

Keynote lecture "Strategies to identify bacterial consortia that colonize the root and increase plant productivity" - **Jeff Dangl**

19:00 - 21:00

Dinner **C**

Day 13 – Friday, April 6th

08:30 - 08:45
08:45 - 09:00

Briefing SR 1
Troubleshooting SR 1

Morning session: Metagenome approaches for microbiota studies SR 1

09:00 - 10:30
10:30 - 10:45
10:45 - 12:45
12:45 - 13:00
13:00 - 14:00
14:00 - ...

Hands-on: Overview of tools for de novo metagenome assembly
and taxonomic assignment (binning)
Coffee break SR 1
Hands-on: Measuring selective pressure in metagenomes
Closing remarks Paul Schulze-Lefert
Lunch C
Farewell