Preparing samples for fungal community sequencing

Department of Forest Mycology and Plant Pathology
Uppsala BioCenter
Swedish University of Agricultural Sciences

PhD course

June 9th-14th 2014

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This course protocol is compiled by Karina Clemmensen and Katarina Ihrmark
Department of Forest Mycology and Plant Pathology, SLU, June 2014

Schedule

Time	Monday 9/6	Tuesday 10/6	Wednesday 11/6	Thursday 12/6	Friday 13/6
09:00	**Registration & Course program**	**Lecture: meta-barcoding** Karina Clemmensen	**Lecture: meta-genomics** Germán Bonilla-Rosso	**Lecture: RNA sequencing** Taina Pennanen	**Laboratory: DNA pooling & quality control**
09:30	**Fika & Speed-dating**				
10:00		**Fika**	**Fika**	**Fika**	
10:15	**Lecture: Sampling design** Jan Stenlid	**Lecture: DNA extraction** Malin Elfstrand	**Lecture: HTS platforms** Olga Vinnere Pettersson . **Discussions** Pros & cons platforms	**Lecture: Primer design & Multiplexing** Björn Lindahl . **Discussions**	
11:00	**Excursion with field sampling** . **Packed lunch**	**Lecture: qPCR & PCR inhibition** Åke Olsson			
11:30					
12:00		**Lunch**	**Lunch**	**Lunch**	**Lunch**
12:30					
13:00		**Laboratory: DNA extraction, purification**	**Laboratory: DNA quantification, PCR tests**	**Laboratory: Producing multiplexed DNA amplicons**	**Lab continued**
13:30					
14:00					**Course wrap-up**
14:30					
15:00					
15:30					**Closing**
16:00	**Laboratory: sample preparation**				
16:30					
17:00					
17:30					
18:00					
18:30					
19:00					

Rooms:	
	Lectures: BioC A241
	Lab work: BioC BÖL4

Field excursion teachers:

Anders Dahlberg, Jan Stenlid, Ariana Kubartová, Dmitry Schigel, Karina Clemmensen

Laboratory teachers:

Monday: Karina, Ariana

Tuesday: Katarina Ihrmark, Anna Berlin, Kerstin Varenius, Karina

Wednesday: Karina, Erica Sterkenburg, Andreas Hagenbo

Thursday: Hanna Friberg, Diem Nguyen, Hanna Millberg

Friday: Ariana, Karina, Katarina

Course aim

This compendium is compiled for the PhD course entitled "How to prepare a sample for high-throughput sequencing of fungal communities" arranged by Department of Forest Mycology and Plant Pathology on behalf of the Nordic-Russian Boreal Forest Biodiversity Education Network funded by SIU and co-funded by the NEFOM (North European Forest Mycologists) network, NordForsk. The course focuses on how environmental samples should be obtained and further processed for next generation sequencing.

By the end of the course participants should be well acquainted with all the steps in the preparation of environmental samples for high-throughput sequencing of fungal communities, as well of the theoretical background and main pitfalls.

The course is held 9-13th June 2014 at the BioCenter, Swedish University of Agricultural Sciences, Uppsala. Teachers include researchers at Dept. Forest Mycology and Plant Pathology as well as invited presenters.

Course experimental design

The idea behind the setup of the field and lab sample preparation in this course is to compare the variation in fungal communities originating from different steps in the sample preparation pathway. If we are able to prepare good DNA samples, we will sequence the final course mix!

The experimental setup for the whole course:

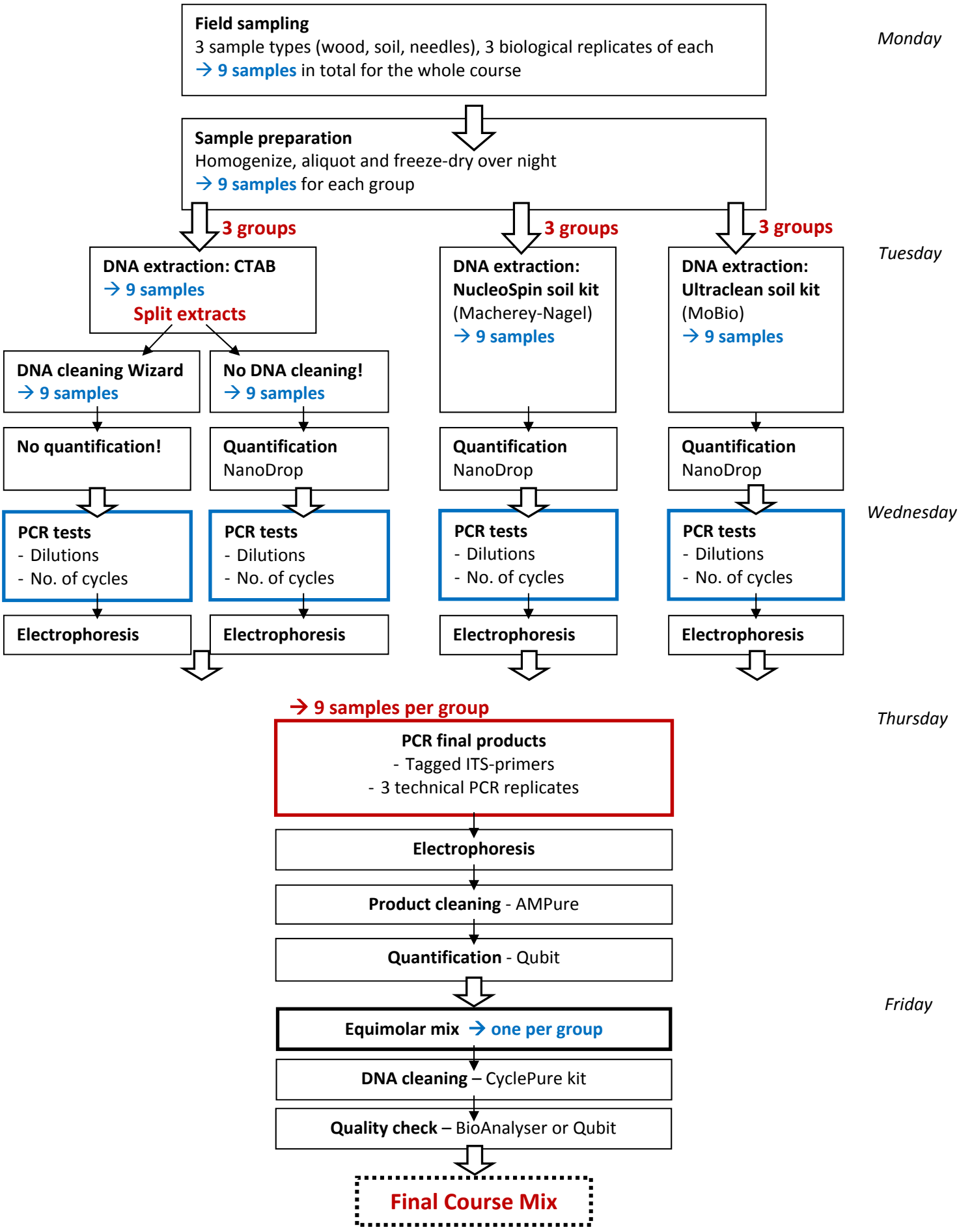
Testing:	***Source of variation:***	***No. of samples***
"The biological factor"	3 sample types	3 samples
	3 biological replicates of each	3x3 = 9 samples
"The extraction factor"	3 DNA extraction methods	3x3x3 = 27 samples
"The human factor"	3 working groups per method	3x3x3x3 = 81 samples

Our hypothesis could be that: ***Biological factor >> Extraction factor >> Human factor***

General safety notes

- Make sure that you know where the **fire protection** equipment, the **first aid** equipment and the **emergency showers** are located
- Always use lab **coat and gloves**
- Always work with **chloroform** in a fume hood!!
- Read the **Risk assessment boxes** before and after each exercise!

Flow chart of practical work



Field sampling and sample preparation

Risk class:
☑No risk ☐Low risk ☐Medium risk ☐High risk
Risk type:

Field sampling

Material
Plastic bags
Marker pens
Small spade
Soil sampler
Wood sampler/drill
Knifes

Procedure
Sample three independent biological replicates of each of three samples types and bring them back in plastic bags:

- Humus
- Needles/leaves
- Wood

→ **9 samples** in total for the whole course

Sample preparation

Material
9 boxes
Scissors
Glass beads and nuts
2 ml screw cap extraction tubes
Racks for 2 ml tubes
Small spoons
Parafilm
Vacuum freeze-drier (BioC basement)

Procedure
The whole course:

1. Place the 9 field samples in the 9 boxes
2. Homogenize the samples by cutting with scissors (humus and needles) and/or shaking

Form 9 groups of two persons (from this point, work in these groups):

3. Add three glass beads (soil) or two nuts (needles/wood) to every screw cap tube with a spoon. Include an 'extraction blank' that it treated as the samples from this point and on, but without sample added.

4. Take about 1 ml subsample of each of the 9 homogenized samples and place in marked 2 ml screw cap tubes.
5. When all groups are ready, place the tubes with the lids NOT fully tightened in the freeze-drier overnight. Use Parafilm to keep the lids in place.
6. Follow the freeze-drier instruction manual placed next to the machine.

Risks and protection

Chemicals:

Organisms:

Radioactivity:

Other:

General notes on DNA extraction and purification

Although the development of new extraction protocols and kits is fast the fundamentals of DNA extraction remains the same: DNA must be purified from cellular material in a manner that prevents degradation and enables amplification. DNA extraction protocols for fungi and bacteria can vary depending on the substrate (e.g. soil, plant, water) of interest. Unlike animals, both bacteria and fungi have sturdy cell walls that need to be broken in order to access the cell content. For this, initial grinding of the freeze-dried samples breaks down cell wall material and allows access to DNA, while harmful cellular enzymes remain inactivated. In our protocol we will pulverize soil, litter and wood samples in a bead beater. Once the sample has been sufficiently homogenized, it can then be re-suspended in a suitable buffer, such as the CTAB extraction buffer. By combining dry and wet bead beating with heating of the samples we ensure that DNA is released also from organisms with thick cell walls that are likely to be attached to soil particles. In order to purify DNA, insoluble particles are removed through centrifugation while soluble proteins and other material are removed through extraction with chloroform and centrifugation. Thereafter DNA must be precipitated with 2-propanol or ethanol from the aqueous phase and washed thoroughly with ethanol to remove contaminating salts. Finally the purified DNA is re-suspended and stored in TE buffer or sterile double deionised water (ddH_2O). This method has been shown to give sufficiently intact genomic DNA from fungal tissue and bacteria for the PCR reaction to work, although further DNA purification is needed for some sample types. Also, to further purify very phenol rich materials, 2% PVP can be added to the extraction buffer.

DNA extraction:
In this course we will test three different extraction procedures developed for difficult samples such as soil: The above mentioned **CTAB protocol** and two different kits developed especially for soil to gain the highest DNA yields with as little PCR inhibitor contaminations as possible: the **NucleoSpin Soil kit** from Macherey-Nagel and the **UltraClean soil DNA Isolation kit** from Mo Bio laboratories.

DNA purification:
To test whether further purification of the extracted DNA is necessary, we will use the **Wizard DNA purification kit** from PROMEGA on a sub-sample of the CTAB extracts.

DNA quantification and quality control:
To check the purity and quantity of the DNA, the samples are analysed spectrophotometrically by the **NanoDrop**. The background reading with this method is quite high and therefore this quantification method is not recommended for exact concentration measurements at <5 ng/ul. For exact quantification of the final PCR products we will use the **Qubit dsDNA assay**, which is based on a fluorophore binding to the double stranded DNA and is thus much more specific at low DNA concentrations. The final DNA mixes will also be run on a 'gel on a chip' on the **Bioanalyzer**, which will give both the size distribution, concentration and the molarity of the DNA.

PCR product purification:
We will combine two different methods to purify the final PCR products to remove leftovers from the PCR mix and to get rid of all smaller (unwanted) DNA fragments, like primer dimers: the **AMPure kit** (on all separate PCR products) and the **CyclePure kit** on the final sample of mixed PCR products.

DNA extraction - CTAB protocol

Risk class:
☐No risk ☐Low risk ☑Medium risk ☐High risk
Risk type: Chemical See specifications in box at page 20

Material

CTAB extraction buffer (3% CTAB, 150 mM Tris-HCl (pH 8±0), 2.6 M NaCl, 2 mM EDTA)
Chloroform
Isopropanol (2-propanol)
70% Ethanol
ddH_2O (MilliQ)
Microcentrifuge tubes (1.5 ml)
Pipette tips (1 ml and 200 µl filter tips)
Heating block pre-heated to 65°C
Bead beater machine
Vortex
Centrifuge
Ice

Procedure

1. Remove field samples from the freeze-drier and tighten the lids.
2. Homogenise dried samples in the Bead beater machine set at low speed (5.0) and time (10 s). Repeat once more time if needed.
3. Transfer 50 mg of the material plus the glass beads or nuts into new tubes, discard the rest.
4. Add ready-made CTAB extraction buffer (1000 µl). For your information the protocol for CTAB preparation is included below.
5. Run samples in the Bead Beater machine once again. The CTAB will froth like detergent.
6. Incubate for 30 min at 65°C in a heating block and vortex every 10 min.
7. Spin down particles in table top centrifuge at 10,000 *rpm* for 5 min.
8. Remove the top of the mixture, avoiding particles as much as possible, to a new marked microcentrifuge tube using a pipette and filter tips (500 µl).
9. Add chloroform (500 µl). **All work with chloroform must be carried out in a fume hood.**
10. Shake the samples vigorously by hand.
11. Centrifuge the mixture at 10,000 *rpm* for 5 min.
12. Remove 400 µl of the upper phase (supernatant) to a new marked microcentrifuge tube. Take care not to get any chloroform or interphase with the supernatant.
13. (Repeat chloroform step if necessary.)
14. Mix the supernatant with 600 µl isopropanol (1.5 x volume) and leave on ice for 20 min.
15. Centrifuge at 13,000 *rpm* for 10 min.
16. Discard the supernatant by gently pouring it off in the sink or beaker. **The DNA should now be in the pellet!!**

17. Wash the pellet with 70% ethanol (500 µl). Centrifuge at 6,500 *rpm* for 5 min. Discard ethanol by pouring it off.
18. (Optional. Carefully remove remaining liquid with a pipette.)
19. Let the pellet air dry by putting tubes upside down with a paper towel under for 5-30 min.
20. Re-suspend the pellet in 100 µl ddH_2O. Gently tap tube to dissolve pellet.

Preparation of the CTAB buffer

Note: Make new bottle of CTAB each day to obtain best extraction efficiency and to avoid contaminating samples.

3% CTAB 50 ml
Dissolve in microwave oven:
1.5 g CTAB (Hexadecyltrimethylammonium bromid)
16.3 ml MilliQ water

Mix with:
7.5 ml 1 M TRIS-HCl
26 ml 5 M NaCl
0.2 ml 0.5 M EDTA

TRIS-HCl 1M pH8
Dissolve:
121.1 g Trisbas
800 ml MilliQ water

Adjust pH to 8 with about 42 ml concentrated HCl
Adjust volume to 1 l
Sterilize by autoclaving, check pH

EDTA 0.5M
Dissolve and stir with magnet:
186.1 g disodium ethelenediaminetetracetate x 2 H_2O (EDTA)
800 ml MilliQ

Adjust pH to 8 with about 20 g NaOH
Adjust volume to 1 l
Sterilize by autoclaving, check pH

DNA purification – Wizard DNA clean-up system (PROMEGA)

Risk class:
☐No risk ☐Low risk ☑Medium risk ☐High risk
Risk type: Chemical
See specifications in box at page 20

Material

- 80% isopropanol (2-propanol)
- pre warmed (65–70°C) deionized water or TE buffer
- disposable 3 ml Luer-Lok syringes

Notes:

- Thoroughly mix the Wizard DNA clean-up resin before removing an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 37°C for 10 minutes. The resin itself is insoluble. Cool to 25–30°C before use.
- The binding capacity of 1 ml of resin is approximately 20µg of DNA.
- The sample volume must be between 50 and 500µl. If the sample volume is less than 50µl, bring the volume up to at least 50µl with sterile water. If the sample volume is >500µl, split the sample into multiple purifications.

Procedure

1. Use one Wizard minicolumn for each sample. Remove and set aside the plunger from a 3 ml disposable syringe. Attach the syringe barrel to the Luer-Lok extension of each minicolumn.
2. Add 1 ml of Wizard DNA clean-up resin to a 1.5 ml microcentrifuge tube. Add **HALF** of your DNA extract (50µl) to the clean-up resin and mix by gently inverting several times.
3. Pipet the Wizard DNA clean-up resin containing the bound DNA into the syringe barrel. Insert the syringe plunger slowly and gently push the slurry into the minicolumn with the syringe plunger. Discard the flow-through.
4. Detach the syringe from the minicolumn and remove the plunger from the syringe. Reattach the syringe barrel to the minicolumn. To wash the column, pipet 2 ml of 80% isopropanol into the syringe. Insert the plunger into the syringe and gently push the solution through the minicolumn. Discard the flow-through.
5. Remove the syringe barrel and transfer the minicolumn to a 1.5 ml microcentrifuge tube. Centrifuge the minicolumn for 2 minutes at 10,000 x *g* to dry the resin.
6. Transfer the minicolumn to a new microcentrifuge tube. Apply 50 µl of pre-warmed (65–70°C) water or TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) to the minicolumn and wait 1 minute. (The DNA will remain intact on the minicolumn for up to 30 minutes.) Centrifuge the minicolumn for 20 seconds at 10,000 x *g* to elute the bound DNA fragment.
7. Remove and discard the minicolumn. The purified DNA may be stored in the microcentrifuge tube at 4°C or –20°C.

DNA extraction – NucleoSpin soil kit (Macherey-Nagel)

Risk class:
☐No risk ☐Low risk ☑Medium risk ☐High risk
Risk type: Chemical See specifications in box at page 20

Material

- 96-100% ethanol
- 1.5 ml microcentrifuge tubes
- Pipette tips

Before starting the preparation

- Check Lysis Buffer SL1 or SL2 for precipitated SDS. Dissolve any precipitate by incubating the buffer at 30–40 °C for 10 min and shaking the bottle every 2 min.

Notes:

- NucleoSpin® Soil is suitable for processing 250–500 mg of fresh sample material. However, do not fill the NucleoSpin® Bead Tube higher than the 1 mL mark (including the ceramic beads) to ensure sufficient head space for an efficient mechanical disruption.
- Usually a reduction of starting material helps to improve the lysis efficiency and to increase the purity of the DNA. Very dry material can soak up large volumes of lysis buffer. In this case, either reduce the amount of sample material or add additional lysis buffer up to the 1.5 ml mark of the NucleoSpin® Bead Tube.
- Ideally, for a new sample material both lysis buffers Buffer SL1 and SL2 should be tested with and without adding Enhancer SX. These initial four preparations will help you to find the ideal lysis condition for your special soil composition.

Procedure

1. Prepare sample
    - Remove field samples from the freeze-drier and tighten the lids.
    - Homogenise dried samples in the Bead beater machine set at low speed (5.0 m/s) and time (10s). Repeat one more time if needed.
    - Weigh 50 mg of the material into NucleoSpin® Bead Tube containing the ceramic beads.
    - Add 700 µl Buffer SL2.

1. Adjust lysis conditions
    - Add 150 µl Enhancer SX and close the cap.
      Note: Enhancer SX ensures the highest possible DNA yield. It can, however, also promote the release of humic acids.

2. Sample lysis
    - Obtain mechanical lysis by running samples in Bead beater at 5 m/s for 30 s
      Alternatively: Attach the NucleoSpin® Bead Tubes horizontally to a vortexer, for example, by taping and vortex the samples at full speed at room temperature (18–25 °C) for 5 min.

3. Precipitate contaminants
    - Centrifuge for 2 min at 11,000 x *g* to eliminate the foam caused by the detergent.

Note: The clear supernatant can be transferred to a new collection tube (not provided) prior to the following precipitation. This might result in more consistent yields from prep to prep and is highly recommended for carbonate containing samples.

- Add 150 µl Buffer SL3 and vortex for 5 s.
- Incubate for 5 min at 0–4 °C. Incubate on ice.
- Centrifuge for 1 min at 11,000 x *g*.

4. Filter lysate
   - Place a NucleoSpin® Inhibitor Removal Column (red ring) in a Collection Tube (2 ml, lid).
   - Load up to 700 µl clear supernatant of step 4 onto the filter.
   - Centrifuge for 1 min at 11,000 x *g*.
     Note: With very wet samples (e.g., sediments) the volume of clear supernatant of step 4 can exceed 700 µl significantly. In this case transfer the NucleoSpin® Inhibitor Removal Column to a new collection tube (not provided) and load the remaining supernatant. Centrifuge for 1 min at 11,000 x g. Combine the flow-throughs.
   - Discard the NucleoSpin® Inhibitor Removal Column.
   - If a pellet is visible in the flow-through, transfer the clear supernatant to a new collection tube (not provided).

5. Adjust binding conditions
   - Add 250 µl Buffer SB and close the lid.
   - Vortex for 5 s.

6. Bind DNA
   - Place a NucleoSpin® Soil Column (green ring) in a Collection Tube (2 ml).
   - Load 550 µl sample onto the column.
   - Centrifuge for 1 min at 11,000 x *g*.
   - Discard flow-through and place the column back into the collection tube.
   - Load the remaining sample onto the column.
   - Centrifuge for 1 min at 11,000 x *g*.
   - Discard flow-through and place the column back into the collection tube.

7. Wash and dry silica membrane

   1st wash:
   - Add 500 µl Buffer SB to the NucleoSpin® Soil Column.
   - Centrifuge for 30 s at 11,000 x *g*.
   - Discard flow-through and place the column back into the collection tube.

   2nd wash:
   - Add 550 µl Buffer SW1 to the NucleoSpin® Soil Column.
   - Centrifuge for 30 s at 11,000 x *g*.
   - Discard flow-through and place the column back into the collection tube.

   3rd wash:
   - Add 700 µl Buffer SW2 to the NucleoSpin® Soil Column.
   - Close the lid and vortex for 2 s. Centrifuge for 30 s at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

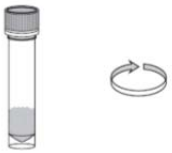
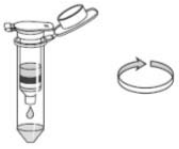
   4th wash:
   - Add 700 µl Buffer SW2 to the NucleoSpin® Soil Column.
   - Close the lid and vortex for 2 s. Centrifuge for 30 s at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see section 6.2 for ordering information.

8. Dry silica membrane
    - Centrifuge for 2 min at 11,000 x *g*.
      If for any reason, the liquid in the collection tube has touched the NucleoSpin® Soil Column after the drying step, discard flow-through and centrifuge again.

9. Elute DNA
    - Place the NucleoSpin® Soil Column into a new microcentrifuge tube (not provided).
    - Add 50 µl Buffer SE to the column.
    - Do not close the lid and incubate for 1 min at room temperature (18–25 °C). Close the lid and centrifuge for 30 s at 11,000 x *g*.

NucleoSpin® Soil

1	**Prepare sample**		NucleoSpin® Bead Tube 250–500 mg sample material 700 µL SL1 **or** SL2
2	**Adjust lysis conditions**		150 µL Enhancer SX
3	**Sample lysis**		Horizontally vortex 5 min at RT or use other homogenizers according to manufacturers protocol
4	**Precipitate contaminants**		11,000 x *g*, 2 min 150 µL SL3 Vortex 5 s 0–4 °C, 5 min 11,000 x *g*, 1 min
5	**Filter lysate**		Load supernatant on NucleoSpin® Inhibitor Removal Column (red ring) 11,000 x *g*, 1 min
6	**Adjust binding conditions**		250 µL SB Vortex 5 s
7	**Bind DNA**		Load 550 µL sample on NucleoSpin® Soil Column (green ring) 11,000 x *g*, 1 min Load remaining sample 11,000 x *g*, 1 min
8	**Wash silica membrane**		1st 500 µL SB 11,000 x *g*, 30 s 2nd 550 µL SW1 11,000 x *g*, 30 s 3rd 700 µL SW2 Vortex 2 s 11,000 x *g*, 30 s 4th 700 µL SW2 Vortex 2 s 11,000 x *g*, 30 s
9	**Dry silica membrane**		11,000 x *g*, 2 min
10	**Elute DNA**		30–100 µL SE RT, 1 min 11,000 x *g*, 30 s

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DNA extraction – UltraClean soil DNA Isolation kit (MO BIO)

Risk class:			
☐No risk	☐Low risk	☑Medium risk	☐High risk
Risk type: Chemical		See specifications in box at page 20	

Material

- Pipette tips (1 ml, 200 µl filter tips)

Before starting the preparation

- If Solution S1 is precipitated, heat solution to 60°C until dissolved before use.
  What's happening: Solution S1 contains SDS. If it gets cold, it will precipitate. Heating to 60˚C will dissolve the SDS. The Solution S1 can be used while it is still warm.
- Shake to mix Solution S3 before use.
- Make sure the 2 ml Bead Solution Tubes rotate freely in your centrifuge without rubbing.

Procedure

1. Remove field samples from the freeze-drier and tighten the lids.

2. Homogenise dried samples in the Bead beater machine set at low speed (5.0 m/s) and time (10s). Repeat one more time if needed.

3. Weigh **50 mg** of the material into the 2 ml **Bead Solution Tubes** provided.
   What's happening: The soil sample or fecal sample has now been loaded into the Bead Tube. This is the first part of the lysis procedure. The Bead Solution is a buffer that will disperse the soil particles and begin to dissolve humic acids.

4. Gently vortex to mix.
   What's happening: This step mixes the sample and Bead Solution.

5. Add 60 µl of **Solution S1** and invert several times or vortex briefly.
   What's happening: Solution S1 contains SDS. This is a detergent that aids in cell lysis. The detergent breaks down fatty acids and lipids associated with the cell membrane of several organisms.

6. Add 200 µl of **IRS Solution** (Inhibitor Removal Solution).
   What's happening: IRS is a proprietary reagent designed to precipitate humic acids and other PCR inhibitors. Humic acids are generally brown in color. They belong to a large group of organic compounds associated with most soils that are high in organic content.

7. Vortex horizontally at maximum speed for 10 minutes. Eventually, secure tubes horizontally on a flat-bed vortex pad with tape.
   Note: Alternatively, obtain mechanical lysis by running samples in Bead beater at 5 m/s for 30 s
   What's happening: The method you use to secure tubes to the vortex is critical. We have designed the vortex adapter as a simple tool that keeps tubes tightly attached to the vortex. It should be noted that although you can attach tubes with tape, often the tape becomes loose and not all tubes will shake evenly or efficiently. This may lead to inconsistent results or lower yields. The use of the vortex adapter is highly recommended for maximum DNA yields. Mechanical lysis is introduced at

this step. The protocol uses a combination of mechanical and chemical lysis. By randomly shaking the beads, they collide with one another and with microbial cells causing them to break open.

8. Make sure the 2 ml **Bead Solution Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
   What's happening: Particulates including cell debris, soil, beads, and humic acids, will form a pellet at this point. DNA is in the liquid supernatant.

9. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Depending upon soil type and amount, expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

10. Add 250 µl of **Solution S2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
    What's happening: Solution S2 contains a protein precipitation reagent. It is important to remove contaminating proteins that may reduce DNA purity and inhibit downstream applications for the DNA.

11. Centrifuge the tubes for 1 minute at 10,000 x *g.*

12. Avoiding the pellet, transfer entire volume of supernatant to a clean **2 ml Collection Tube** (provided).
    What's happening: The pellet at this point contains residues of humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

13. Shake to mix Solution S3 before use. Add 1.3 ml of **Solution S3** to the supernatant and vortex for 5 seconds.
    Note: High volume of solution will touch the rim of the tube. Take care when handling tube.
    What's happening: Solution S3 is a DNA binding salt solution. DNA binds to silica in the presence of high salt concentrations.

14. Load approximately 650 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute.

15. Discard the flow through, add the remaining supernatant to the **Spin Filter**, and centrifuge at 10,000 x *g* for 1 minute. Repeat until all supernatant has passed through the **Spin Filter**.
    Note: Depending on soil type, a total of up to four loads for each sample processed may be required.
    What's happening: DNA is selectively bound to the silica membrane in the spin filter device. Almost all contaminants pass through the filter membrane, leaving only the desired DNA behind.

16. Add 300 µl of **Solution S4** and centrifuge for 30 seconds at 10,000 x *g*.
    What's happening: Solution S4 is an ethanol based wash solution used to further clean the DNA that is bound to the silica membrane in the spin filter. This wash solution removes residues of salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.
    Note: You can wash more than one time to further clean DNA if desired. In some cases where soils have very high humic acid content, it will be beneficial to repeat this wash step.

17. Discard the flow through from the **2 ml Collection Tube**.
    What's happening: This flow through is just waste containing ethanol wash solution and contaminants that did not bind to the silica spin filter membrane.

18. Centrifuge again at 10,000 x *g* for 1 minute.
    What's happening: This step removes residual Solution S4 (ethanol wash solution). It is critical to remove all traces of wash solution because it can interfere with downstream applications for the DNA.

19. Carefully place **Spin Filter** in a new clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution S4** onto the **Spin Filter**.
    What's happening: Once again it is important to avoid any traces of the ethanol based wash solution.

20. Add 50 µl of **Solution S5** to the center of the white filter membrane.
    What's happening: Placing the Solution S5 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in more efficient release of the desired DNA.

21. Centrifuge at 10,000 x *g* or 30 seconds.
    What's happening: As the Solution S5 (elution buffer) passes through the silica membrane, DNA is released, and it flows through the membrane, and into the collection tube. The DNA is released because it can only bind to the silica spin filter membrane in the presence of salt. Solution S5 is 10mM Tris pH 8.0 and does not contain salt.

22. Discard the **Spin Filter**. DNA in the tube is now ready for any downstream application. No further steps are required.

23. We recommend storing DNA frozen (-20°C to -80°C). **Solution S5** contains no EDTA.

DNA quantification - NanoDrop

Material
Your DNA samples
ddH_2O
Pipette tips (10 µl tips)
Kimwipes
NanoDrop machine (Dept Forest Mycology and Plant Pathology)

Procedure

1. With the swing arm in the down position, open the NanoDrop software by a double click on the NanoDrop icon (not on the pdf help file icon).
2. From the first window select nucleic acid.
3. Start by cleaning the instrument on both the upper and lower pedestals using lens-cleaning tissue (Kimwipes). Add 2 µl ddH_2O onto the lower pedestal, close the sample chamber by lowering the swing arm carefully. Click "Ok" and you should then see the message "Initializing Spectrometer – please wait". When this message disappears, the instrument will be ready for use.
4. From the "Sample Type" list, select "DNA-50".
5. Blank measurement: Load your blank sample (1.5 µl ddH_2O OR elution buffer) onto the lower pedestal, close the sample chamber and click on the "Blank" button on the screen.
6. Once the blank has been analysed, confirm that the blank has yielded a reproducible zero, by analysing a blank as though it was a sample. The result should give a spectrum that varies no more than 0.003 Absorbance Units (AU) from the stored blank value (± 1.5 ng/µl for nucleic acid).
7. Clean the instrument between each measurement by simply wiping the sample from both the upper and lower pedestals using Kimwipes.
8. For each measurement incl. blanks, type in a "Sample ID/name" for later sample identification.
9. Sample measurement: Type in sample name, load your sample (1.5 µl), and select the "Measure" button on the measurement screen.
10. Repeat step 7-9 for the rest of your samples.
    Note! If there is an error message appearing try with re-measuring the sample (there may be an air bubble), run new blanks and/or try diluting the sample further.

What is happening? DNA concentration in the final eluate can be calculated from its absorption maximum at 260 nm (A_{260}) based on the fact that an absorption of $A_{260} = 1$ corresponds to 50 µg/ml double stranded DNA. However, this calculation assumes the absence of any other compound that absorbs UV light at 260 nm. Any contamination with, for example, RNA, protein, or especially humic substances significantly contributes to the total absorption at 260 nm and therefore leads to an overestimation of the real DNA concentration.

DNA The ratio of absorbance at 260/280, and 260/230 nm is used to assess the purity of DNA. Ratios of about 1.8 to 1.7, respectively, are generally accepted as "pure" for DNA. If the ratios are appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 230 or 280 nm. See Fig. below.

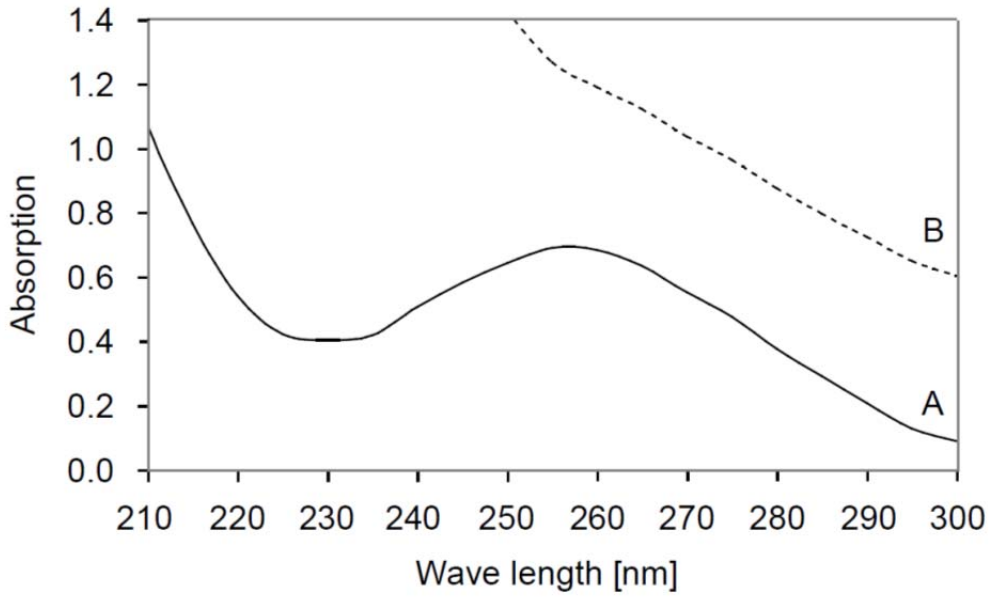


Figure 2: UV-VIS quantification of A) pure DNA and B) contaminated DNA
A) 7.7 µg in 100 µL, 1.84 A_{260}/A_{280}, 1.71 A_{260}/A_{230}
B) 9.3 µg in 100 µL, 1.35 A_{260}/A_{280}, 0.27 A_{260}/A_{230}

Example from the NucleoSpin (Macherey-Nagel) protocol.

Risks and protection

Chemicals: Chloroform is a strong organic solvent. All work with chloroform must be carried out in a fume hood, wearing gloves and a lab coat. Waste chloroform must be kept in fume hood and should not be poured out in the sink. If chloroform is inhaled, make sure that the person gets fresh air. If a large amount of chloroform is spilled outside the fume hood, open fume hoods completely and press the "force venting" button, leave the room and contact staff.

CTAB is corrosive. Avoid skin contact.

All buffers could include mildly hazardous or flammable solvents. Handle them carefully. Avoid skin contact and use gloves at all times.

Organisms:

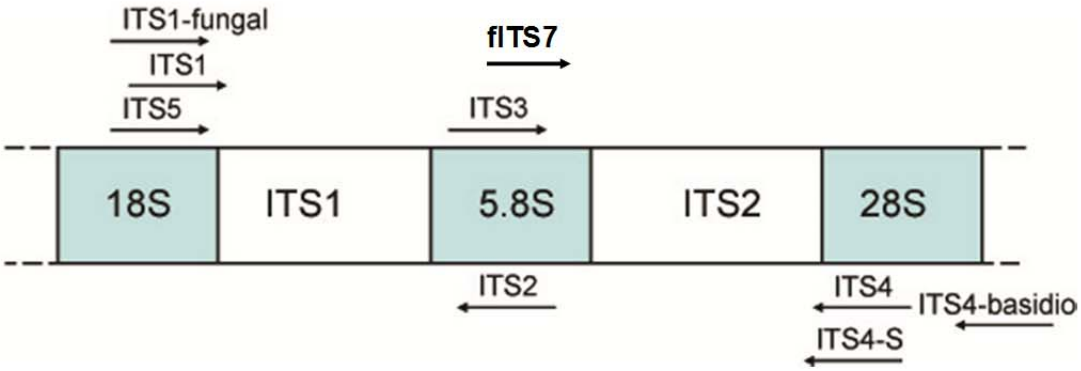
Radioactivity:

Other:

General notes on PCR amplification

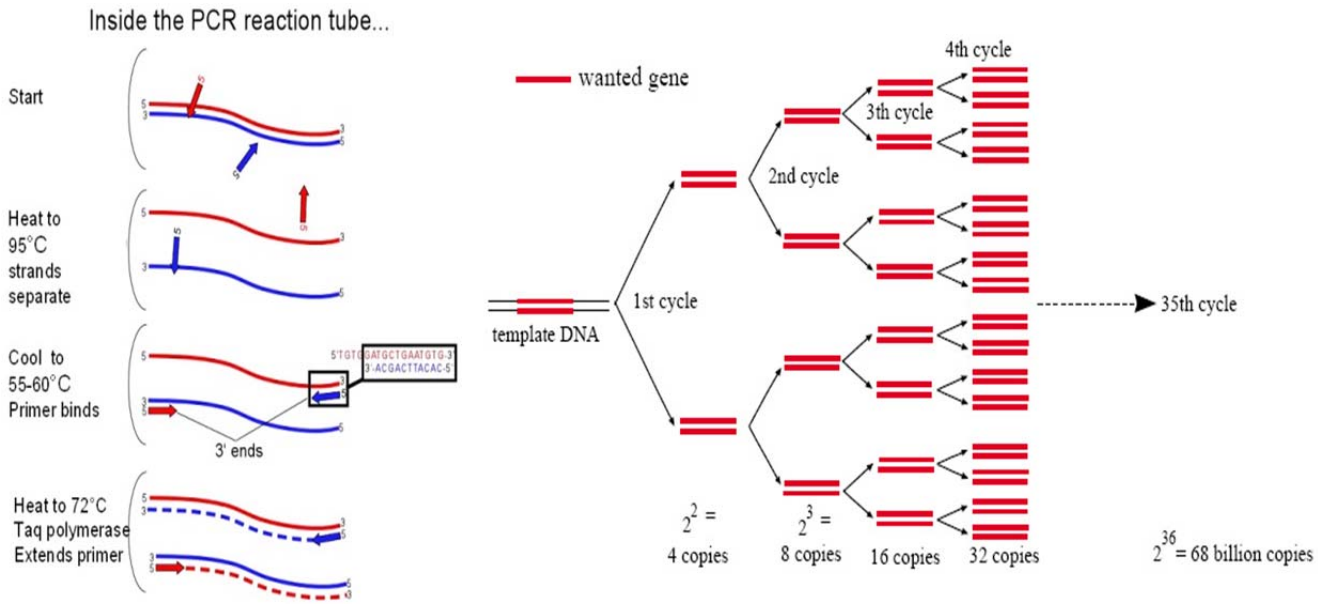
The purpose of a PCR (Polymerase Chain Reaction) is to make a large number of copies of a gene or other part of the genome. This is necessary to yield enough starting template from the extracted DNA for further sequence analysis. During this practical we will amplify the fungal ITS2 region to analyse the fungal communities.

The **Internal Transcribed Spacer** (ITS) region of nuclear ribosomal DNA (rDNA) is now perhaps the most widely sequenced DNA region in fungi, and has been chosen as the bar code for fungal species identification. However it is not optimal for all fungal groups. The ITS region, separating 18S, 28S and 5.8S coding regions of the rDNA gene, is present in high copy numbers allowing easy amplification of the region from total DNA. It has typically been most useful for molecular systematics at the species level because of its high rate of polymorphism between species. The ITS region is separated into ITS1 and ITS2, both immediately flanking the 5.8S gene sequence, with the former upstream and the latter downstream of that sequence.



ITS primer map. Fungal specific primer fITS7 (forward) and universal primer ITS4 (reverse) will be used.

There are three major steps in a PCR, which are repeated for 20 to 40 cycles. This is done on an automated thermal cycler, which can heat and cool the tubes with the reaction mixture and DNA template in a very short time. Because both DNA strands are copied during PCR, there is an exponential increase of the number of copies of the gene.



Schematic figure of the principles of a PCR.

1. **Denaturation** at 94°C: During the denaturation, the double strand melts apart to single stranded DNA, and all enzymatic reactions stop.
2. **Annealing** at 57°C: The primers form temporary ionic bonds with the single stranded template. The more stable bonds last a little bit longer (primers with a good fit) and on that piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.
3. **Extension** at 72°C: This is the ideal working temperature for the polymerase. The primers, where a few bases are already built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again due to the higher temperature. The bases that are complementary to the template, are coupled to the primer on the 3' side. The polymerase adds dNTP's from 5' to 3', reading the template from the 3' to the 5' side; bases are added complementary to the template.

Determining dilutions and number of PCR cycles - PCR tests

In order to get a representative community of sequences in the final sample sent for sequencing - i.e. the same as in the original sample - it is pivotal to determine both the optimal dilution of your template DNA and the optimal number of cycles of PCR amplifications to run. The samples should be diluted to overcome any inhibition of the PCR reaction present in the extracts. The number of PCR cycles should allow the reaction to reach (the middle of) the phase of exponential increase of product, but not to enter the 'saturated' phase in which the community could be altered due to e.g. primer, dNTP or enzyme limitation.

Optimal dilutions and cycle numbers can be tested either with normal PCR or with quantitative real-time PCR (qPCR, see figure). Here we will only use normal PCR.

We will test the DNA dilutions: **x10, x100 and x1000 (Wizard cleaned), 5, 0.5 and 0.05 ng/µl (not Wizard cleaned)**
Cycle numbers: **22, 25, 28 and 31**

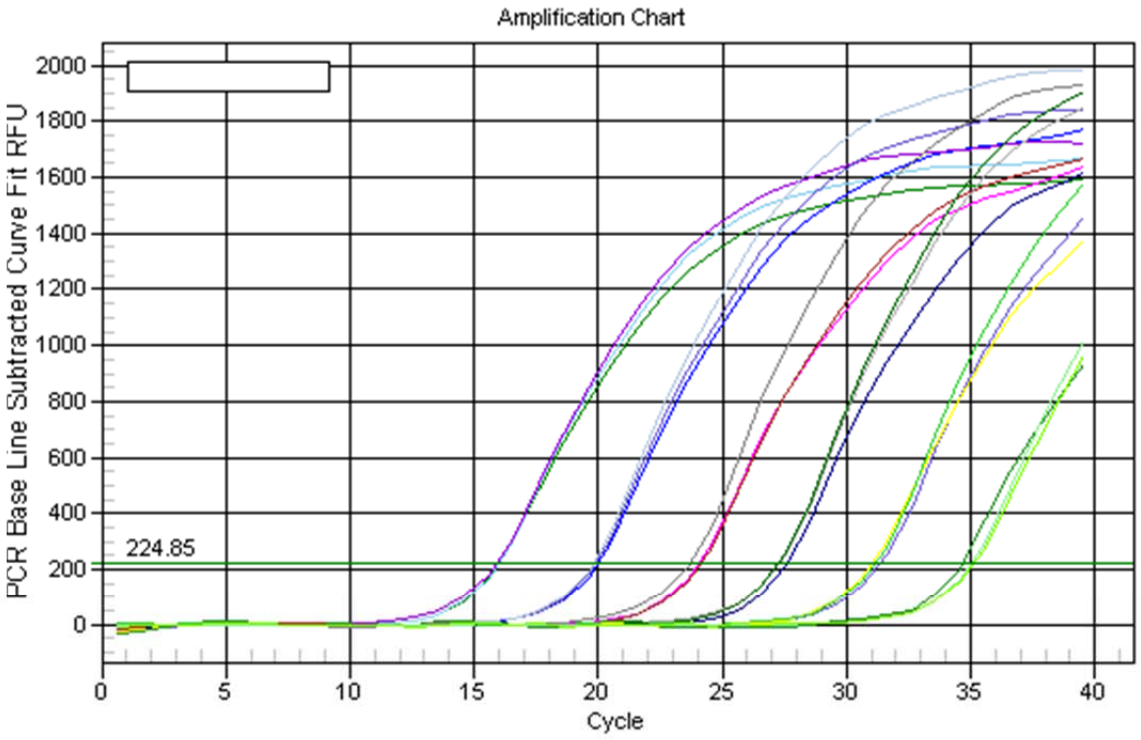


Figure showing standards with known ITS copies run in a quantitative real-time PCR (qPCR). The optimal number of cycles for producing amplicons for sequencing can likewise be determined by qPCR. The total number of ITS copies in a sample can also be determined by relating the sample to a standard curve like this. However, you need to test for and eventually account for any PCR inhibition in the sample.

PCR dilution and cycle number tests

Risk class:
☐No risk ☑Low risk ☐Medium risk ☐High risk
Risk type: Chemical See specifications in box at page 39

Material

Marker pen
PCR-strips of 8 tubes each
Pipette tips (200 µl and 10 µl filter tips)
Ice
Taq polymerase, 'DreamTaq' (stock 5U/µL) (keep on ice – it is an expensive enzyme!)
Reaction Buffer (supplied with the *Taq* polymerase)
dNTPs (2 mM each of dATP, dCTP, dGTP and dTTP in a mixture)
$MgCl_2$ (stock 25 mM)
ITS primers with ID tags, supplied in a mixed stock, **one tag needed per group!**
ITS4 (8bpIDtag-TCCTCCGCTTATTGATATGC), 0.3 µM in final mix
fITS7 (8bpIDtag-GTGARTCATCGAATCTTTG), 0.5 µM in final mix
ddH_2O
Your DNA samples

fITS7 mix	Stock, µM	Final, µM	1 µl react	50 µl reaction	50 µl reactions
				x1 reaction	x____ reactions
ddH_2O			0.165	8.25	
Buffer	x10	x1	0.1	5	
dNTPs	2000	200	0.1	5	
$MgCl_2$	25000	750	0.03	1.5	
DreamTaq polymerase	5u/µl	0.01	0.005	0.25	
ITS primers with tags			0.1	5	
SUM			0.5	25	
Master mix			0.5	25	25
Template DNA			0.5	25	25
Reac vol.			1	50	50

Procedure

1. Choose one representative DNA extract from each of the **three** sample types (humus, litter, wood). If you have used the Wizard cleaning kit, then chose both with and without Wizard cleaning → 6 samples in total (3 Wizard, 3 non-Wizard)
2. Dilute the Wizard-cleaned samples x10, x100 and x1000 times with ddH_2O in Eppendorf tubes → 9 dilutions

   Dilute the non-Wizard-cleaned samples to 5, 0.5 and 0.05 ng/µl based on the NanoDrop measurement with ddH_2O in Eppendorf tubes → 9 dilutions

Dilution formula: C1 x V1=C2 x V2
C: concentration, V: volume, 1: initial, 2: final

3. Prepare a master mix with sufficient material for 9 or 18 reactions plus three-five extra samples – there will be pipetting losses. Also, remember to run negative controls (blanks) with water added instead of DNA template; run one blank per 9 samples. Fill out the table above to establish how much you need to take of each ingredient. Label your PCR tubes and fill out the table below to organize your samples.
4. Take out the reagents from the freezer, defrost them and put them on ice.
5. Pipet everything except your template DNA into a microcentrifuge tube. Vortex gently and quickly spin down the mixture using the table mini centrifuge.
6. Aliquot 25 µl into each PCR tube.
7. Add your template (25 µl) using new pipette tips for each sample.
8. Cap the tubes properly, spin down in a mini centrifuge and put tubes into the thermal cycler (PCR machine) and start the ITS57 program. Remember to add information of the reaction volume and the desired number of cycles to run.

ITS57 program, cycle conditions:

95°C 5 min	
95°C 30 sec	22-31 cycles
57°C 30 sec	
72°C 30 sec	
72°C 7 min	

9. Press ‘PAUSE’ when the wanted cycle numbers are reached (22, 25, 28, 31) and take out an aliquot of 10 µl from each sample into a new tube. Run the negative controls with samples at 31 cycles.

10. Run the PCR products on an agarose gel.

Samples run in test PCRs (not Wizard-cleaned):

Tube number	Sample	Dilution	No. cycles	Product on gel?

Samples run in test PCRs (Wizard-cleaned):

Tube number	Sample	Dilution	No. cycles	Product on gel?

Electrophoresis – visualization of DNA on agarose gel

Before the PCR product is used in further applications, it has to be checked to determine whether any product has formed, whether they are of the right size and whether there is one or multiple bands.

In electrophoresis, different sizes of DNA molecules are arranged and separated, according to their sizes. When an electrical field is applied to the agarose gel, DNA molecules, with their negatively charged phosphate groups, will migrate from the negatively charged start point, towards the positively charged end. Smaller DNA molecules will move more quickly in the gel than larger ones. Consequently, after some time (30 min), you get a spatial separation of smaller DNA molecules, close to the positively charged end point, and larger molecules close to the negatively charged start point.

Not every PCR is successful. There is for example a possibility that the quality of the DNA is poor, that the primers does not fit, or that there is too much or too little starting template. It is further possible that there is a product, but of a different size than the expected size. In that case, one of the primers probably fits on another part of the gene than the supposed part. It is also possible that both primers fit on a totally different part of the genome, yielding more bands on the gel.

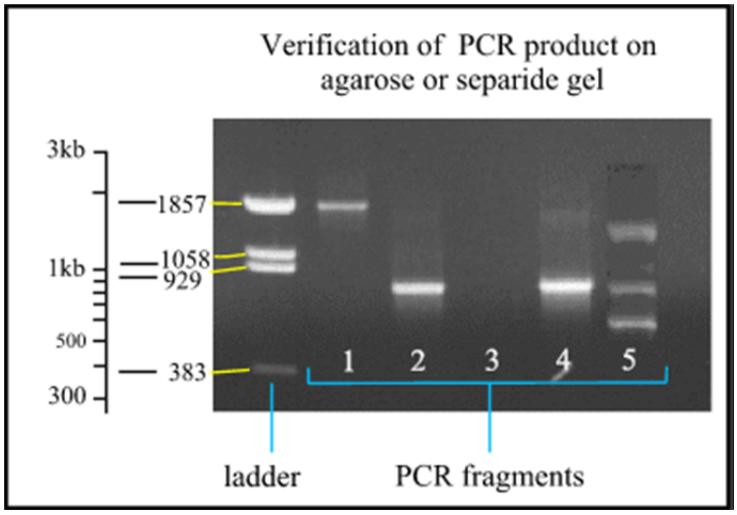


Figure 3. The PCR products obtained after PCR will be separated on a 1% agarose gel, stained with NancyDye and visualized under blue light.

Running an agarose gel

Risk class:			
☐No risk	☑Low risk	☐Medium risk	☐High risk
Risk type: Chemical		See specifications in box at page 39	

Material

Agarose
1X SB buffer
Nancy dye
Pipette tips (yellow)
DNA size standard GR-mix
Gel tray system and comb
Tape
Gel documentation kit with UV or blue light (at dept. Mykopat)

Procedure

1. You can load up to 80 samples in a gel with 2 combs each with 40 teeth: Weigh 2.2 g of agarose and add it to an E-flask (500 ml), and add 220 mL 1X SB to the flask. Add 4 µl Nancy dye.
2. Melt the agarose in a microwave oven. Make sure there are no bubbles or unmelted agarose left after microwaving.
3. Allow the melted agarose to cool to about 60°C or cool enough to handle with your hands. While the agarose is cooling tape up the gel tray (15 cm) and insert the comb (30 teeth), then pour the slightly cooled agarose into the tray and allow it to solidify about 30 minutes. When the agarose has solidified carefully remove the comb and tape.
4. Load 3 µl of ladder in the wells at the ends of the gel. Then load 5 µl of your samples into the wells.
5. Place the tray with the gel in an electrophoresis cell and run the gel at about 250V 400mA for 20 minutes.
6. Stop the power aggregate, remove your gel and take a photo under UV or blue light in the gel documentation kit (situated at Dept. Forest Mycology and Plant Pathology, 3rd floor).

PCR – final products

Risk class:
☐No risk ☑Low risk ☐Medium risk ☐High risk
Risk type: Chemical See specifications in box at page 39

Material

Marker pen
PCR-strips of 8 tubes each
Pipette tips (200 µl and 10 µl filter tips)
Ice
Taq polymerase, 'DreamTaq' (stock 5U/µL) (keep on ice!)
Reaction Buffer (supplied with the *Taq* polymerase)
dNTPs (2 mM each of dATP, dCTP, dGTP and dTTP in a mixture)
$MgCl_2$ (stock 25 mM)
ITS primers with ID tags, supplied in a mixed stock: **11 tags needed per group!**
ITS4 (8bpIDtag-TCCTCCGCTTATTGATATGC), 0.3 µM in final mix
fITS7 (8bpIDtag-GTGARTCATCGAATCTTTG), 0.5 µM in final mix
ddH_2O
Your DNA samples

fITS7 mix	Stock, µM	Final, µM	1 µl react	50 µl reaction	50 µl reactions
				x1 reaction	x____ reactions
ddH_2O			0.165	8.25	
Buffer	x10	x1	0.1	5	
dNTPs	2000	200	0.1	5	
$MgCl_2$	25000	750	0.03	1.5	
DreamTaq polymerase	5u/µl	0.01	0.005	0.25	
SUM			0.4	20	
Master mix			0.4	20	20
ITS primers with tags			0.1	5	5
Template DNA			0.5	25	25
Reac vol.			1	50	50

Procedure

1. Evaluate your test PCR results!

   For each sample type - evaluate:

   Which dilution worked the best => which gave strongest bands on the gel?

   At which cycle number did you get product?

2. Dilute your 9 samples to the dilutions decided upon. You will need at least enough diluted sample to run three PCR reactions with 25 µl diluted sample in each.
3. Calculate how many PCR reactions you want to run.

→ Number of samples x 3 PCR replicates of each

4. Prepare a master mix with sufficient material for a few of extra samples – there will be pipetting losses. Remember to run PCR negative controls (blanks) with water added instead of DNA template. Fill out the table to establish how much you need to take of each ingredient.
5. Take out the reagents from the freezer, defrost them and put them on ice.
6. Pipette everything but the tagged ITS4 primers and your template DNA into a microcentrifuge tube. Vortex gently and quickly spin down the mixture using the table mini centrifuge.
7. Aliquot 20 µl master mix into each PCR tube.
8. Add 5 µl of the tagged ITS primer mix to each PCR tube using new pipette tips for each sample. **Use different ITS-tags for each sample as well as for extraction and PCR blanks, but same for three PCR replicates of each sample. NOTE DOWN CAREFYLLY WHICH ITS-TAG GOES WITH WHICH SAMPLE IN THE TABLE BELOW!!**

9. Add your template (25 µl) using new pipette tips for each sample. Do three PCR replicates of each.
10. Cap the tubes properly, spin down in a mini centrifuge and put tubes into the thermal cycler (PCR machine) and start the ITS57 program. Remember to add information on the reaction volume and the desired number of cycles to run.

    Cycle conditions, ITS57 program:

    95°C 5 min
    95°C 30 sec
    57°C 30 sec
    72°C 30 sec
    } 22-31 cycles
    72°C 7 min

11. If your samples need to be run at different cycle numbers - press 'PAUSE' when the wanted cycle number is reached and take out samples at each stop. Save the samples in the fridge. Run the negative controls with samples at the highest cycle number.
12. Run the PCR products on an agarose gel!
13. Continue with the good PCR products to the AMPure cleaning step!

Samples run in final PCRs:

Tube number	**Sample**	**Dilution**	**ITS-tag**	**No. cycles**

Purification of PCR products with AMPure (Beckman Coulter)

Risk class:			
☐No risk	☑Low risk	☐Medium risk	☐High risk
Risk type: Chemical		See specifications in box at page 39	

The PCR-products must be purified to get rid of salts, unincorporated dNTPs and unused primers. The AMPure kit consists of small magnetic beads that bind DNA. By using a magnet to retain the beads (with the DNA) it is possible to wash the DNA. Once the beads have been washed and dried the DNA can be eluted from the beads.

Material

AMPure magnetic bead solution
Magnet
PCR-plate
70% EtOH
Kitchen-roll paper
Elution buffer
ddH2O
PCR-products
Pipette tips (yellow)
Multipipette
Dispensing tray
Oven (37^{o}C)

Procedure

1. Add 81 µl AMPure magnetic bead solution to each sample of 45 µl.
2. Transfer the PCR-product/bead mix to a PCR-plate.
3. Incubate at room temperature 3-5 min.
4. Place the plate on the magnetic plate. Incubate 5-10 min.
5. Keeping the plate on the magnet, turn the plate upside-down and try to get rid of the liquid. The plate can be gently hit against a table with kitchen tissue to absorb the liquid.
6. Add 200 µl 70% EtOH to each well, incubate for 30 sec at room temperature. Get rid of the liquid as in step 5.
7. Repeat step 6. This time it is important to get rid of as much liquid as possible. Hit the plate hard several times against the table, until no drops appear on the kitchen-roll paper. **Don't forget to keep the plate in the magnetic plate!**
8. Let the plate dry at 37°C for 30-60 min. The magnetic plate is not needed at this stage.
9. Remove the plate from the magnet. Add 60 µl elution buffer to each well, cover with plastic foil, vortex and spin down.
10. Place the plate on the magnet before pipetting the supernatant. Alternatively the magnetic beads can be pelleted by centrifugation at 3000 rpm for 10 sec.

DNA quantification – Qubit dsDNA HS Assay Kit (Life Technologies)

Risk class:			
☐No risk	☑Low risk	☐Medium risk	☐High risk
Risk type: Chemical		See specifications in box at page 39	

Material

Thin-walled, clear 0.5 ml PCR tubes
Post-PCR pipettes – 10 µl and 1000 µl
The Qubit components A, B, C, D
Centrifuge w/ plate adaptor (at dept. Mykopat)
Qubit instrument (at dept. Mykopat)

Note: The HS (high sensitivity) assay is highly selective for double-stranded DNA (dsDNA) over RNA and is accurate for initial sample concentrations from 10 pg/µl to 100 ng/µl. The assay is performed at room temperature, and the **signal is stable for 3 hours**. Common contaminants, such as salts, free nucleotides, solvents, detergents, or protein are well tolerated in the assay.

Procedure

1. All reagents should be allowed to adjust to room temperature.
2. Set up the number of 0.5 ml tubes you will need for standards and samples. The assay requires 2 standards.
3. Make the working solution by diluting the dsDNA HS reagent (component A) 1:200 in dsDNA HS buffer (component B) in a Falcon tube. Prepare 200 µl per sample/standard, plus for one extra sample to account for pipetting losses.

   → Mix 199 µl of buffer (x number of samples) with 1 µl of reagent (x number of samples)
4. Load 190 µl of the working solution into each of the tubes used for standards.
5. Add 10 µl of each standard (C and D) to the appropriate tube and mix by vortexing 2–3 seconds, being careful not to create bubbles.

   Note: Careful pipetting is critical to ensure that exactly 10 µl of each standard is added to 190 µl of working solution.
6. Load 197 µl working solution into individual assay tubes.
7. Add 3 µl of your samples to assay tubes containing the working solution and mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µl.
8. Centrifuge the samples at 3000 rpm for 10 sec to get rid of air bubbles
9. Allow all tubes to incubate at room temperature for 2 minutes.
10. On the Home Screen of the Qubit Fluorometer, press **DNA**, and then select **dsDNA High Sensitivity** as the assay type. The Standards Screen is automatically displayed.
11. On the Standards Screen, press **Yes** to run a new calibration.

12. Running a New Calibration: Insert the tube containing Standard #1 in the Qubit Fluorometer, close the lid, and press **GO**. Also press **START** on the computer screen. The reading will take approximately 3 seconds. Remove Standard #1.
13. Insert the tube containing Standard #2 in the Qubit Fluorometer, close the lid, and press **GO**. Remove Standard #2.
14. Insert a sample tube into the Qubit Fluorometer, close the lid, and press **GO**.
15. Upon the completion of the measurement, the result will be displayed on the screen. Note down the result in the table below. If time allows you can also get the Qubit machine to do this calculations directly after each measurement. Alternatively see calculation below.
16. Repeat sample readings until all samples have been measured.

Calculating the concentration of dsDNA in your sample
The Qubit Fluorometer gives values for the Qubit dsDNA HS assay in ng/ml. This value corresponds to the concentration after your sample was diluted into the assay tube. To calculate the concentration of your sample, use the following equation:

Concentration of your sample = QF value × (200 µl /x µl)
Where: QF value = the value given by the Qubit
x = the number of µl of sample you added to the assay tube

Equimolar mix of PCR products

There are several high-throughput sequencing technologies available at the moment. Most studies on fungal communities during the past five years have used 454-pyrosequencing (Roche). A whole 454 sequencing plate using the Titanium chemistry gives about 1.000.000 sequence reads, of up to about 600bp length. A plate is typically subdivided into 2-16 regions; however you will have fewer reads the more you physically split up a plate. In each region one sample (DNA pool) can be run. However, the 454-sequencing chemistry is on it's way out of the market, as cheaper alternatives with much higher sequence outputs are available, such as the Illumina and Ion torrent technologies.

The fungal ITS2 sequences produced have a maximum length of about 500 bp, and one possibility is to sequence our samples using the Illumina MiSeq alternative giving 20 mill. reads of 600bp length (for about one-tenth of the price as for 1 mill. 454 reads).

To use any of the these techniques optimally to cover amplicons from many samples, multiplexing using sample-specific tags is necessary, and the samples that you want to run in one region should be pooled. At dept. Forest Mycology and Plant Pathology we have about 100 ITS-primers (both ITS4 and fITS7) extended with different 8bp identification tags.

In order to get approximately the same number of sequences from each sample, an equimolar mix of all samples should be prepared. However, the ITS2 amplicons that have been amplified contain templates varying in length between about 250 and 500 bp, and measuring the molar concentration of all samples (e.g on the BioAnalyzer) would be costly in labour and money. A good approximation can be obtained by mixing the same amount of DNA (ng) from each sample as measured by Qubit.

Calculate µl needed from each sample to get the same amount of DNA in the mix. If possible, mix >50-100 ng DNA from each of your PCR reactions (if all PCRs worked you will have 27 PCR amplicons plus 6 blanks (3 DNA prep and 3 PCR blanks!). If some PCR products are very weak you may have to scale amounts of these samples down relative to the rest of the samples, i.e. take fewer ng of these samples. Calculate the amount to be pooled from each sample like this:

µl sample to be pooled
= (DNA amount to be pooled, e.g.50 ng) / (DNA concentration in the sample, ng/µl)

The final amount needed per DNA pool that is send for sequencing is about **1 µg of PCR product** (depending on sequencing platform). In the final cleaning of the group-mix with Omega cycle pure, typically half of the DNA is lost. Therefore, the amount of DNA in each of the group-mixes should optimally be at least **200-400 ng** of DNA.

Samples in final DNA pool to be sequenced:

Sample	**Qubit, ng/µl**	**Sample, ng/µl**	**DNA to pool, ng**	**Sample to pool, µl**

Clean PCR products with Cycle-Pure Kit (Omega)

Risk class:			
☐No risk	☑Low risk	☐Medium risk	☐High risk
Risk type: Chemical		See specifications in box at page 39	

Material

Microcentrifuge
1.5 ml microcentrifuge tubes
2 ml collection tubes
DNA Mini Column (a small tube with a filter bottom)
ddH_2O
Timer
Buffer CP
DNA Wash Buffer (ethanol diluted)
Pipette tips (yellow and red)

Procedure

1. Determine the volume of your PCR reaction. Transfer the sample into a clean 1.5 ml tube and add 6 volumes of Buffer CP. For example, if your PCR mix is 100 µl, add 600 µl of Buffer CP
2. Vortex thoroughly to mix. Briefly spin the tube to collect any drops from the inside of the lid.
3. Place a DNA Mini Column into the 2 ml collection tube.
4. Add the mixed sample from step 2 to the DNA Mini Column and centrifuge at 13,000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the DNA Mini Column back into the same collection tube. **OBS: The column can only hold 700 µl at a time. If you have more sample than this, you have to load your sample on the same column multiple times and repeat the centrifugation and discard of the flow-through.**
5. Add 700 µl of DNA Wash Buffer (ethanol diluted) and centrifuge at 13,000 x g for 1 minute. Discard the flow-through liquid and place the DNA Mini Column back into the same collection tube.
6. Add 500 µl of DNA Wash Buffer and centrifuge at 13,000 x g for 1 minute. Discard the flow-through liquid and place the DNA Mini Column back into the same collection tube.
7. Centrifuge the empty DNA Mini column for 2 min at maximal speed (≥13,000 x g) to dry the column matrix.

   Note: Do not skip this step; it is critical for the removal of ethanol from the DNA column.
8. Place the DNA Mini column into a clean 1.5 ml microcentrifuge tube. Depending on the desired concentration of the final product, add 30-50 µl of ddH_2O directly onto the centre of column matrix. Incubate at room temperature for 2 minutes. Centrifuge for 1 min at 13,000 x g to elute the DNA. This eluates approximately 80-90% of bound DNA.
9. Go on to with the Bioanalyzer protocol, or store samples at -20 °C until further use.

PCR product quantification and quality – BioAnalyzer (Agilent Tech)

Risk class:			
☐No risk	☑Low risk	☐Medium risk	☐High risk
Risk type: Chemical		See specifications in box at page 39	

Material

DNA Chip
Agilent DNA 7500 Assay Kit
BioAnalyzer instrument (Dept Forest Mycology and Plant Pathology)

Procedure

1. Allow the gel-dye mix equilibrate to room temperature for 30 min before use.
2. Put a new DNA chip on the chip priming station.
3. Pipette 9.0 µl of gel-dye mix in the well marked G.
4. Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
5. Press plunger until it is held by the clip.
6. Wait for exactly 30 s then release clip.
7. Wait for 5 s. Slowly pull back plunger to 1 ml position.
8. Open the chip priming station and pipette 9.0 µl of gel-dye mix in the wells marked G.
9. Pipette 5 µl of marker (green) in all 12 sample and ladder wells. Do not leave any wells empty.
10. Pipette 1 µl of DNA ladder (yellow) in the well marked with a ladder.
11. In each of the 12 sample wells pipette 1 µl of sample (used wells) or 1 µl of de-ionized water (unused wells).
12. Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).
13. Run the chip in the Agilent 2100 bioanalyzer within 5 min.
14. The result will appear in the screen!

Risks and protection

Chemicals:

Nancy-520 dye and Qubit and Bioalalyser kit components contain DMSO, an organic solvent that can go through skin. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care. Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples. Handle the DMSO stock solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

SB buffer includes boric acid. Boric acid is considered hazardous at a level above 5%. In this lab, we are below this threshold (0.2%) but be careful anyway.

AMPure contains sodium azide, which is toxic. Be careful and use gloves.

All buffers could include mildly hazardous solvents. Handle them carefully.

Organisms:

Radioactivity:

Other:

GOOD LUCK!!