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# Illumina metabarcoding protocol for the study of fungi in marine sediments

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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

The study of fungi in marine sediments has received a growing attention in the recent years. Recent advances in metagenomics have allowed the discovery of a wide diversity of fungi in deep-sea environments, although standardized methods are needed to process the samples and to analyze the data. This protocol is a guidance to obtain good quality samples and subsequent ITS1 (Internal Transcribed Spacer 1) amplicons to be prepared for sequencing by Illumina platform. The collection of marine sediments is described, as well as the processing of the samples in laboratory; also, pertinent controls that should be included during all the processing steps to identify potential sources of contamination that could affect the sequencing and bias the interpretation of results are suggested.

# Materials

## Equipment

| | Name | Brand | Catalog number |
|---|---|---|---|
| | Gas burner | | |
| | Scalpel | | |
| | Pipettes with different volume capacity | Gilson | |
| | Sterile scalpel blades | Miltex | 4-311 |
| | Timer | VWR | 62344-904 |
| | Corning Pinnacle 530 pH meter | Thermo Scientific | |
| | Analytical balance | Mettler Toledo | AX105 |
| | Vortex Genie® 2 | MoBio | |
| | Vortex Adapter for 24 (1.5–2.0 ml) tubes | Qiagen | 13000-V1-24 |
| | Centrifuge | Eppendorf | 5427-R |
| | NanoDrop™ Lite | Thermo Fisher Scientific | ND-LITE |
| | Qubit 3.0 Fluorometer | Life technologies | Q33216 |
| | UVP UK-plug UV2 PCR Workstation | UVP | 95-0439-02M |

## Materials and reagents

| | Name | Brand | Catalog number |
|---|---|---|---|
| | 10 mL syringes (five per core) | | |
| | Sucrose (molecular biology grade) | Sigma | S0389 |
| | EDTA (prepare at 0.5 M) | Faga Lab | 6381-92-6 |
| | NaCl (prepare at 5M) | Golden Bell | 26270 |
| | Tris-HCl (prepare at 1M) | J. F. Baker | 4109-01 |
| | Bovine Serum Albumin (BSA) | Amresco | 0332 |
| | Pure Ethyl alcohol (molecular biology grade) | Sigma | E7023 |
| | Isopropanol (molecular biology grade) | Sigma | |
| | Phenol:chloroform:isoamyl alcohol pH 7-8 | Sigma | P2069 |
| | HPLC grade water (sterile) | Fermont | H5052 |
| | GoTaq® DNA Polymerase | Promega | M3005 |
| | 5X Colorless GoTaq® Buffer | Promega | M792A |
| | $MgCl_2$ | Promega | |
| | dNTP mix (10 mM) | Promega | U1511 |
| | Forward primer (PCR 1: sequencing primer + ITS1F) | Sigma | |
| | Forward primer (PCR 1: sequencing primer + ITS2) | Sigma | |

| | | | |
|---|---|---|---|
| | Index i7 (adaptor+index) | Sigma | |
| | Index i5 (adaptor+index) | Sigma | |
| | DNeasy PowerSoil Kit | Qiagen | 12888 |
| | Qubit dsDNA HS Assay Kit | Thermo Fisher Scientific | Q32851 |
| | Just-a-Plate ™ 96 PCR Purification Kit | Charm Biotech | JA-100-2 |
| | SequalPrep™ Normalization Plate Kit | Thermo Fisher Scientific | A1051001 |
| | 0.2 µm Sterile syringe filters | VWR | 514-0073 |
| | 50 mL Conical Centrifuge Tubes | Falcon | 352070 |
| | 15 mL Conical Centrifuge Tubes | Falcon | 352096 |
| | 1.7 mL microcentrifuge tubes | VWR | 85003-294 |
| | 0.5 mL Thin Wall PCR Tubes | Axygen-Corning | PCR-05-C |
| | 0.2 mL PCR Tubes | VWR | 20170-012 |
| | Disposable reagent reservoir | Vista Lab Technologies | 3054-1003 |
| | Aluminum foils for 96 well plates | VWR | 60941-126 |

| | | | |
|---|---|---|---|
| | PARAFILM® M | BRAND | PM996 |
| | Kimwipes Kimtech Science | Kimberly Clark | 34155 |
| | Nitrile gloves | Kirkland | 570413 |
| | 0.1-10 µl Filter pipet tips | VWR | 53510-029 |
| | 1–100 µl Filter pipet tips | VWR | 53510-094 |
| | 100-1000 µl Filter pipet tips | VWR | 16466-004 |
| | 1–200 µl Filter pipet tips | VWR | 53510-102 |
| | 1–40 µl Filter pipet tips | VWR | 53510-016 |

## Before start

During all procedures, several contamination controls must be included, in order to detect contaminant DNA from all the material and samples manipulation. Each control step is indicated at the beginning of the corresponding section.

## Preparation of syringes

1

Note

**Control #1: Keep a microcentrifuge tube containing 1 mL of sterile HPLC grade water open during the syringe preparation. The tube has to be close to the working area, where the syringes are manipulated.**

Note

This step should be done near a gas burner in order to preserve sterility.

Grab a sterile syringe package and open it by the end, where the syringe tip is localized. Avoid moving the plunger.

2 Attach a new scalpel blade to the handle and flame sterilize it by using a gas burner.

3 Cut and remove the tip of the syringe carefully.

4 Close a gain the syringe package and secure it with adhesive tape once the tip is cutoff.

5 Sterilize again the scalpel before cutting a new tip.

## Sampling tubes preparation

6

Note

**Control #2: Keep an open microcentrifuge tube containing 1 mL of sterile HPLC grade water during the sucrose buffer preparation.**

**Control #3: Once the sucrose buffer is prepared and sterilized, keep a microcentrifuge tube with 1 mL of sterile sucrose buffer.**

Open the tubes containing the sterile sucrose buffer near the gas burner.

7 Pipette 5 mL of the sterile sucrose buffer (see recipe) into each of the 15 mL tubes.

Note

Recipe - Sucrose buffer (100 mL)
1. Mix the next reagents:

| | Reagent | Quantity |
|---|---|---|
| | EDTA 0.5 M | 4 mL |
| | NaCl 5 M | 8 mL |
| | Tris-HCl 1 M | 5 mL |
| | Sucrose molecular grade | 25.68 gr |

2. Shake vigorously and add distilled water up to 80 mL.
3. Adjust pH to 9.
4. Fill to final volume (100 mL) with distilled water.
5. Near the gas burner, open the 0.2 µm pore filter, and place it in the syringe.
6. Near the gas burner, filter the buffer through the 0.2 µm pore filter and collect the sterile solution in the 50 mL tubes.

8 Cover the tube with a piece of parafilm, to prevent buffer leaks.

9 All tubes should contain 25% w/v sucrose buffer (stored at room temperature) (Figure 1- next section).

## Sediment sampling

10

Note

Note: Prior to sampling, each tube should be labeled with the station number, date, depth and sub-sampling number, and other information you need to identify the sample.

**Control #4: Keep a microcentrifuge tube containing 1 mL of sterile HPLC grade water open while the samples are prepared for storage.**

**Control #5: Keep a microcentrifuge tube containing 1 mL of sterile sucrose buffer open while the samples are prepared for storage.**

Make a reference mark within the core to split it into two halves.

11 Open five pre-cut syringes (10 mL). Pull the plunger back.

12 Immerse syringes (cylinder) in one half of the core (Figure 1) to take the sample.

13 Remove the syringes and put the first 5 mL (corresponding to 10 cm depth) of sediment by pushing the plunger into 15 mL screw cap tubes, and the remaining 5 mL of sediment (corresponding to the first 5 cm) in another 15 mL screw cap tubes.

14 Shake the tubes to obtain a homogenous sample of buffer and sediment.

15 Seal the tubes with the screw cap and wrap them with parafilm.

16 Store tubes inside Ziploc® type bags at -20°C during the campaign and during the transport to the laboratory.

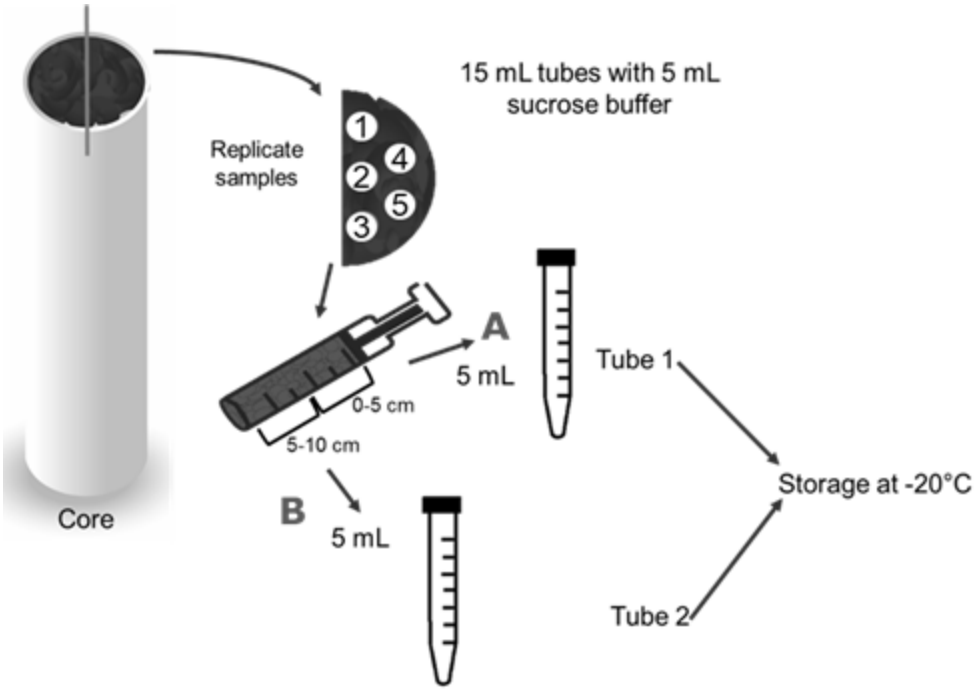


Figure 1.Scheme for sediment sampling and storage.

## Sample preparation

17 Thaw the samples on ice.

18 Centrifuge the samples at maximum velocity for 1 min at room temperature.

19 Discard the supernatant. This step serves to discard the sucrose buffer used to preserve the samples. Store the samples at-80°C until processing.

> Note
>
> Alternatively, the samples can be kept in the sucrose buffer at -20°C in the laboratory, until processing.

## Lyophilization

20 Place tubes containing the corresponding sample uncapped on ice and cover with parafilm the top of each tube.

21 Make a few holes onto the parafilm with a new (sterile) needle.

22 Put samples in the lyophilizer.

23 Check the samples with a sterile needle after 48 hours, before turning off the lyophilizer. The sample is ready if the sediment is totally dry. You can verify this by carefully shaking the sample to make sure the sediment is totally dry.

24 Cap the tubes and store the samples at -80°C until processing.

Note

Samples can be processed without lyophilization

## DNA extraction

25

Note

Modified protocol from “Increasing DNA yield from PowerSoil kit - Info from rep Dr. Tetreault Carlson and from Tips and FAQ portion of MoBio website”.

**Important:** We recommend not to process more than 5-6 samples per round of extraction; it is important to take care of the time that the sediment is in contact with the phenol chloroform reagent.

**Control #6: Keep a microcentrifuge tube containing 1 mL of sterile HPLC grade water open during the sample extraction**.

Prepare the PCR chamber station: First, clean the entire chamber, including the glass walls. Lay all the material that is needed for the DNA extraction inside the chamber. Close the chamber and turn on the UV light for 15 min. Once the chamber is ready, without opening it, turn on the white light and the air circulator. Now the PCR chamber station is ready to be used.

26 Weigh 0.15 grs of lyophilized sample and transfer it into a PowerBead tube. The weighing process must be done with care in order to prevent contamination and cross-contamination between samples.

Note

If the samples are processed without prior lyophilization, take into account that the sediment is mixed with some water/sucrose buffer, and this would increase considerably the volume in the next steps of the DNA extraction procedure. To prevent an increase in volume, remove 100 µL of the PowerBead solution from the PowerBead tube.

27 Return the remaining lyophilized samples to -80°C.

28 Add 60 µL of C1 solution.

29 Add 100 µL of phenol chloroform in the chemical fume hood.

30 Incubate the samples 2 min at room temperature.

31 Lay the samples horizontally in the vortex adapter and vortex for 30 secs (do notexceed this time).

32 Centrifuge the samples for 1 min.

33 Transfer the supernatant to a sterile 2 mL tube.

34 Add 250 µL of C2 solution and vortex for 5 secs. Incubate the samples on ice for 5 min.

35 Centrifuge the samples for 2 min.

36 Transfer 600 µL of the supernatant to a sterile 2 mL tube.

37 Add 200 µL of C3 solution and vortex for 5 secs. Incubate the samples on ice for 5 min.

38 Centrifuge the samples for 3 min.

39 Transfer up to 850 µL of the supernatant to a sterile 2 mL tube.

40 Add 650 µL of C4 solution and 450 µL of absolute ethanol and vortex for 5 secs.

41 Load up to 500 µL onto a column tube. Centrifuge for 30 secs and discard the flow through.

42 Repeat step 18 until the complete sample has been processed.

43 Centrifuge the samples for 2 min and carefully, transfer the column to a new sterile 2 mL tube.

44 Add 600 µL of absolute ethanol and centrifuge for 30 secs. Discard the flow through.

45 Centrifuge the samples for 3 min and discard the flow through.

46 Add 650 µL of C5 solution and centrifuge for 30 secs. Discard the flow through.

47 Centrifuge the samples for 4 min and carefully, transfer the column to a new sterile 2 mL tube.

48 Add 50 µL of C6 solution and incubate at room temperature for 15 min.

49 Centrifuge for 1.5 min.

50 Add 50 µL of C6 solution and incubate at room temperature for 15 min.

51 Centrifuge for 2 min.

52 Discard the column.

53 Visualize the extracted DNA by running an electrophoresis on a 1% agarose gel using 5 µL of DNA for each sample.

## Measuring the genomic DNA with a NanoDropTM Lite Spectrophotometer

54 Once the NanoDrop is on, select the DNA option, and then the option dsDNA (double strain DNA).

55 Carefully lift the upper pedestal.

56 Clean the upper and lower pedestals by pipetting 2 µL of sterile HPLC grade water onto the bottom pedestal and, lower the upper pedestal and wait 2 min. Lift the pedestal and clean the upper and lower pedestal with Kimwipes.

57 Establish a blank by pipetting 1 µL of the blank solution onto the bottom pedestal, lower arm and press Blank.

58 When the measurement is complete, rinse the arm and wipe the blank solution from the upper and lower pedestal using Kimwipes.

59 Repeat step 5 to confirm the Blank. Now you are ready to measure the samples.

60 Measure the sample by pipetting 1 µL (mix the DNA with the pipette) of the sample in the lower pedestal, then, lower the upper pedestal and press Measure. Once ready, the equipment screen displays the results (Figure 2). Lift the pedestal and clean the upper and lower pedestal with Kimwipes and the instrument is ready to measure the next sample.

Note

A total of two or three measures are necessary for each sample, in order to confirm the values of DNA concentration.

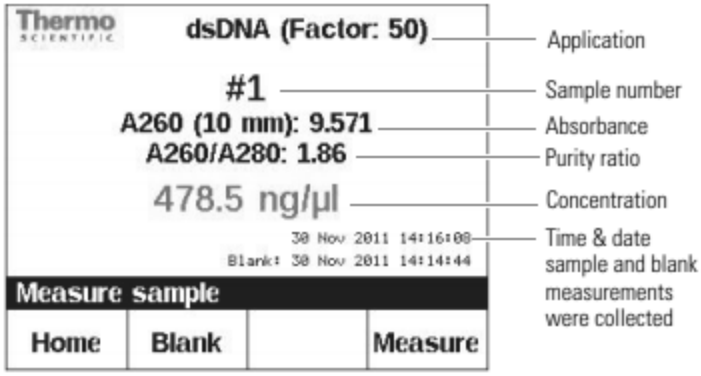


Figure 2. Illustration of NanoDrop™ Lite Spectrophotometer results screen.

| Ratio | Value | Explanation |
|---|---|---|
| A260/A280 | 1.8-2 | Optimal purity DNA |
| | 1.6-1.8 | DNA of acceptable purity |
| | <1.6 | Presence of aromatic compounds |
| | >2.1 | RNA contamination |

Table 1. Nucleic acid purity ratios.

61 Once all samples are measured, repeat step 3.

## Measuring the genomic DNA with Qubit 3.0

62

Note

All steps are conducted at room temperature as recommended in the Qubit™ dsDNA HS Assay Kit user guide.

Be careful to grab the tubes by the lid and try not to touch the rest of the tube, to avoid leaving marks on them.

In a 2 mL microcentrifuge tube prepare the working solution by diluting the Qubit BR Reagent (Component A) 1:200 in the Qubit BR buffer (Component B). Prepare enough working solution for samples and standards. The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of the working solution, and each sample

requires anywhere from 180-199 µL. 3 µL of each sample is enough for the DNA measurement.

63 Prepare the two standards by adding 10 µL of the standard to 190 µL of working solution.
Standard 1: 0 ng/µL
Standard 2: 10 ng/µL

64 Prepare the samples by adding 3 µL of DNA to 197 µL of working solution.

65 Mix the samples and standards by vortexing (2-3 secs) and apply spin just for a few secs.

66 Allow all tubes to incubate at room temperature for two min. Proceed with themeasurement of the DNA:
- Connect the Qubit 3.0.
- On the Home screen of the Qubit® 3.0 Fluorometer, press DNA, then select dsDNA High Sensitivity as the assay type. The “Read standards” screen is displayed. Press Read Standards to proceed.
- Insert the tube containing Standard 1 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard 1.
- Insert the tube containing Standard 2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard 2.
- The instrument displays the results on the Read standard screen.
- Press Run samples.
- On the assay screen, select the sample volume and units:
Press the + or – buttons on the wheel to select the sample volume added to the assay tube (3 µL).
From the dropdown menu, select the units for the output sample concentration (ng/µL).
- Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 secs), remove the sample tube. The instrument displays the results on the assay screen. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration.

## Fungal PCR and library preparation

67

Note

The PCR is aimed to amplify the ITS1 region of rDNA (Figure 2-A). The primers used are ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns, 1993) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'; White et al. 1990).
A two-PCR strategy is followed to obtain the amplicon libraries for Illumina sequencing. The first PCR includes the ITS1F and ITS2 primers attached to a Sequencing primer (Figure 3-B), and the second PCR is designed for the attachment of the Illumina adapter and barcode (identification sequence of each sample) (Figure 3-C).

**Controls 1 to 6 should be processed as the samples, and they should be included in all PCR runs. Besides these controls, the PCR must include an additional negative control, using as template sterile HPLC grade $H_2O$, and a positive control; for instance, a mock community: a mix of genomic DNA extracted from different fungal strains.**

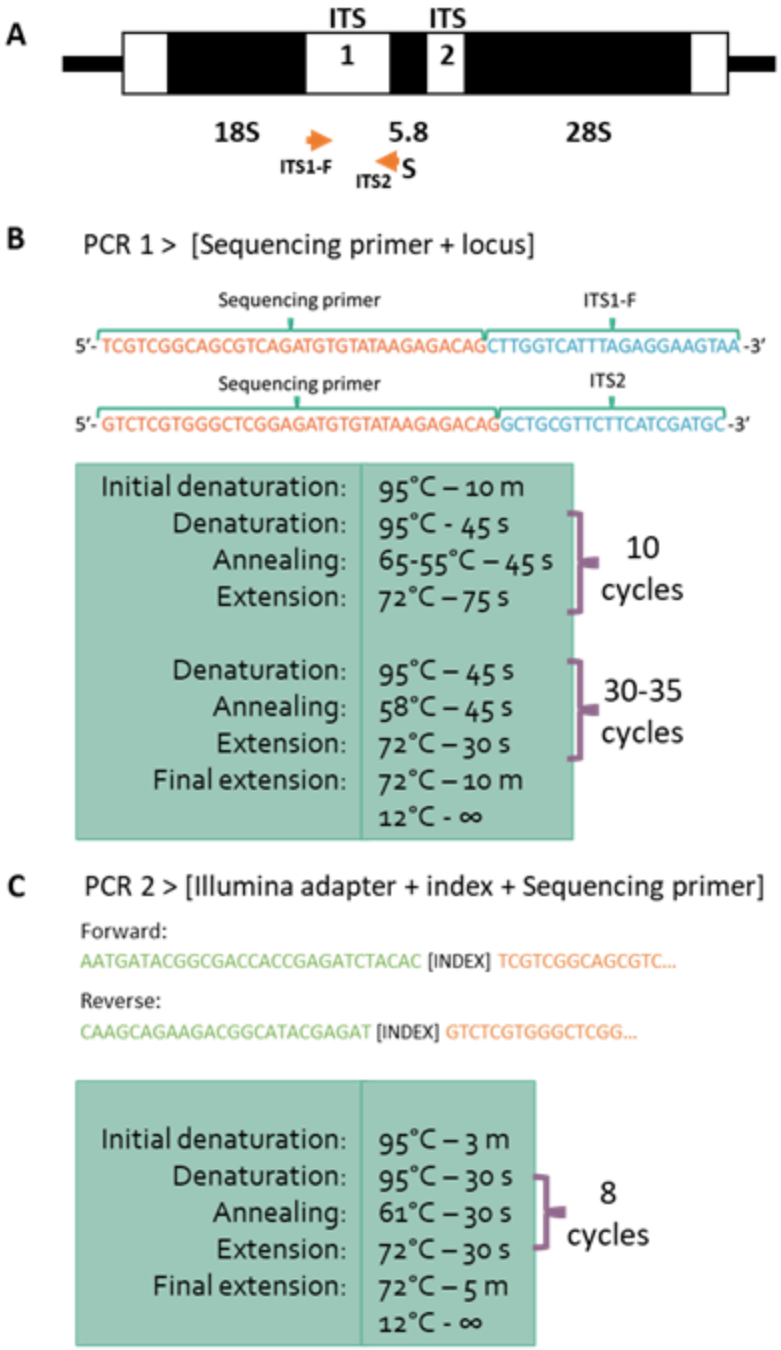


Figure 3. PCR strategy to obtain the amplicon library.

## PCR1: Touchdown PCR for the ITS1 region amplification

68 Prepare the PCR chamber station: First, clean the entire chamber, including the glass walls. Lay all the material that is needed to prepare the DNA and prepare the PCR reactions inside the chamber. Close the chamber and turn on the UV light for 15 min.

Once the chamber is ready, without opening it, turn on the white light and the air circulator. Now the PCR chamber station is ready to be used.

Note

Label all the tubes necessary for all the process.

69 Keep the samples genomic DNA on ice until processing, and adjust the DNA concentration to 2 ng/µL.

70 Prepare a master mix by multiplying the volume of each PCR reagent (Table 2) times the number of samples to be processed and add an extra 10% to account for pipetting errors. The Touchdown PCR program is indicated in Figure 3-B.

71 Run 5µL of PCR product on a 2% agarose gel electrophoresis to check the correct amplification of the desired size fragment (>300 bp).

| Reagent | Concentration | 50 µL final volume - 1 reaction (µL) |
|---|---|---|
| HPLC grade water | ---- | 17.7 |
| BSA | 10 mg/ µL | 4 |
| Buffer | 5x | 10 |
| $MgCl_2$ | 25 mM | 6 |
| dNTP mix | 10 µM | 4 |
| Forward primer | 10 µM | 1.5 |
| Reverse primer | 10 µM | 1.5 |
| Polymerase | 5 U | 0.3 |
| Genomic DNA | 2 ng/ µL | 5 |

Table 2. Master Mix reagents, concentrations and preparation.

## Purification of PCR product

72

Note

From PCR 1, a primer-dimer band is usually obtained (~120 bp) and should be removed, in order to avoid amplification of this undesirable product in the PCR 2 step. To do this, use the Just-a-Plate ™ 96 PCR Purification Kit.

Solutions from the protocol are included in the kit.

Prepare the PCR chamber station. First, clean the PCR chamber, including the glass walls, put and prepare all the material that is needed for the PCR purification inside the PCR chamber. Close the chamber and turn on the UV light during 15 min. Instructions of how to use are on the side of the PCR chamber. Once the chamber is ready, without opening it, turn on the white light and the air circulator. Now the PCR chamber station is ready to use.

73 First time using the kit: Add 96 mL of 100% ethanol to Washing Buffer II (WB2) and mix well and mark bottle that ethanol has been added.

74 Add 100 µL of Binding Buffer (KB5) to and 400 µL Isopropanol (IPP) to 1.5 mL microtube. Mix well by inverting tube 10 times after close the cap.

75 Repeat 8 times, so you have total 8 wells with 45 µL PCR products. Then for each well, add 37 µl of working Binding Buffer (KB5) prepared in step (3) to each well, mix well by pipetting the solution up and down 10 times (use clean pipet tips each time).

76 Cover the plate with a clean adhesive plate cover.

77 Spin the plate for 10 min at 2250 x *g* speed at room temperature (use another plate for balance).

78 Remove the plate cover.

79 Quickly flip over the plate over a waste container and shaking briskly to remove allthe solution in each well.

80 Add 150 µL of washing buffer II to each well. Pipet up and down 2 times. Wait for 30 secs.

81 Repeat steps 8 and 9.

82 Repeat step 8.

83 Blot dry the inverted plate on a piece of clean absorbent paper 3 – 4 times gently.

84 Air dry for 10 min.

85 Add 20 µL of elution buffer to each well, and cover the plate with an adhesive cover, and vortex it for 30 secs. After vortexing, spin the plate for 30 secs to collect all the liquid at the bottom of each well.

86 Load 5 µl into a 1% agarose gel.

## PCR 2: Illumina adapter and barcode ligation to PCR product

87

Note

We have a total of 20 indexes available (i7 index: 12; i5 index: 8). These indexes are sufficient for a total of 96 samples. A certain combination of indexes for each sample should be assigned.

| Reagent | Concentration | 30 µL final volume - 1 reaction (µL) |
|---|---|---|
| HPLC grade water | ---- | 12.55 |
| BSA | 10 mg/ µL | 2.4 |
| Buffer | 5x | 6 |
| $MgCl_2$ | 25 mM | 3.6 |
| dNTP mix | 10 µM | 2.4 |
| Index | 10 µM | 0.9 |
| Index | 10 µM | 0.9 |
| Polymerase | 5 U | 0.25 |
| Purified PCR product | ----- | 1 |

Table 3. Master Mix reagents, concentrations and preparation.

Prepare a master mix by multiplying the volume of each PCR reagent (Table 3) times the number of samples to be processed.
The PCR program is indicated in the Figure 2-C.
Visualize the PCR products by electrophoresis in a 2% agarose gel. Load 5 µL from each sample. Since the ITS region is variable in size, differently sized PCR products, ranging from 300 bp to even 800-900 bp, should be expected.

## PCR purification and normalization

88 For this step, follow the supplier instructions from the SequalPrep™ Normalization Plate Kit.

## Illumina sequencing

89 For the sequencing, the MiSeq Reagent Kit v3 (600-cycle) is used in order to obtain 300 bp for each forward and reverse ends.