Preparation of Fungal ITS Amplicons from Soil Samples for Illumina Amplicon Sequencing

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Abstract

This protocol was used for the molecular profiling of the soil fungal community of two winter wheat fields as a part of a long-term field trial in Germany with following Illumina Amplicon Sequencing. Besides different pre-crops, the effects of soil management and fertilization on the fungal communities were investigated.

Due to different aspects of our protocol, we wanted to ensure reproducing a realistic ratio of the different fungal communities in the soils. The most important steps to conform this demand being the frequently measurements of DNA concentrations at different steps of the protocol, the high numbers of PCRs of each soil sample and the use of the qPCR setting a cut-off for the PCR cycles to avoid an over-amplification of certain PCR-fragments and a loss of detectable biodiversity. The use of the two DNA isolation kits based on the cooperation with our project partner and the exchange of DNAs.

Citation: Loreen Sommermann,Joerg Geistlinger,Jessica Zwanzig,Ingo Schellenberg Preparation of Fungal ITS Amplicons from Soil Samples for Illumina Amplicon Sequencing. **protocols.io**

dx.doi.org/10.17504/protocols.io.nmgdc3w

Published: 12 Mar 2018

Guidelines

Gloves sholud be worn at all the time and changed regularly.

Materials

PowerSoil DNA Isolation Kit 12888-100 by Mobio

Qubit® dsDNA HS Assay Kit Q32854 by Thermo Fisher Scientific

Qubit® 3.0 Fluorometer Q33216 by Thermo Fisher Scientific

GENECLEAN® SPIN KIT 111101400 by MP Biomedicals

Phusion High Fidelity Master Mix with HF Buffer F531L by Thermo Fisher Scientific

MinElute Gel Extraction Kit 28606 by Contributed by users

DNA Gel Loading Dye (6X) R0611 by Thermo Fisher Scientific

GeneRuler 100 bp DNA Ladder SM0241 by Thermo Fisher Scientific

GeneRuler 1 kb DNA Ladder SM0311 by Thermo Fisher Scientific

Roti®-GelStain 3865.1 by Carl Roth

Roti®-Load DNA-orange 1 HP04.1 by Carl Roth

- Orange 50 bp DNA Ladder 25-2300 by Contributed by users
- Midori Green direct MG06 by Contributed by users
- SYBR Green I Nucleic Acid Gel Stain 50513 by Contributed by users

FastDNA Spin Kit for Soil by MP Biomedicals

Protocol

DNA isolation

Step 1.

The total DNA of the soil samples was extracted by using two different DNA isolation kits.

The PowerSoil® DNA Isolation Kit (Mo Bio) and the FastDNA ™ SPIN Kit for Soil (MP Biomedicals) were used for three and one isolations, respectively.

The quality of the extracted DNAs mixed with 6x Loading Dye were checked on 0.8% agarose gels. We combined the DNA of one isolation of the PowerSoil® DNA Isolation Kit with the isolated DNA of the FastDNA ™ SPIN Kit. In summary, we have three DNA isolations of each soil sample.

NOTES

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Please note, we changed the following steps of the protocol from the PowerSoil® DNA Isolation Kit.

We centrifuged each time for 1 min instead of 30 seconds.

The wash step (step 16) was performed twice with each of 250µl.

The DNA was eluted in 80 µl Solution C6 instead of 100µl.

Step 2.

Aliquots of each DNA-Isolation were purified using Gene Clean Spin Kit (MP Biomedicals) following the protocol.

The concentration of the purified and non-purified DNAs was measured with Qubit® 3.0 (invitrogen) and dsDNA HS Assay Kit (invitrogen). The measurement based on 1µl of DNA.

The DNAs were diluted to a concentration of 10ng/µl ensuring a comparable input of template DNA to the following PCRs.

PCR conditions

Step 3.

To avoid over-amplification of specific PCR-fragments and loss of detectable biodiversity, in preliminary experiments the beginning of the exponential PCR phases with soil DNA templates and ITS1 and ITS2 primers were determined by qPCR using 1x SYBR® Green Nucleic Acid Stain (Lonza, Switzerland).

qPCR Master Mix (1 reaction)

10µl	Phusion High-Fidelity PCR Master Mix with HF Buffer (F-531L)	0.4 U	
2µl	forward primers: ITS1-F_KYO2 (ITS1) ITS86R (ITS2)	0.5µM of each primer	Reference [2] Reference [3]
2µl	reverse primers: ITS86F (ITS1) ITS4 (ITS2)	0.5µM of each primer	Reference [3] Reference [1]
2µl	SYBR®Green Nucleic Acid Stain dissolved in TE-buffer (1:1000)		
2-3µl	nuclease-free water		
1-2µl	template DNA (10ng/µl)		
20µl	**total reaction volume**		

Step 4.

Reactions were performed in a Piko Real 96 thermal cycler (Thermo Fisher Scientific) with the following conditions:

qPCR ITS conditions

Step 1	94°C	5 min
Step 2	94°C	15 sec
Step 3	56°C	25 sec
Step 4	72°C	20 sec
number of cycles (from step 2 to step 4) were 37		

Step 5.

According to qPCR results, the cutoffs for the following preparative PCRs were set in the middle of the

exponential phases, which were 22 cycles for ITS1 and 21 cycles for ITS2 primers.

Step 6.

Preparative PCRs were performed with barcoded NGS primers using the standard eight nucleotide barcodes and the primer pairs ITS1F_KYO2 and ITS86R for the ITS1 region and ITS86F and ITS4 for the ITS2 region. The use of sample-specific barcodes allowed for the extraction of sample-specific amplicon reads after Illumina sequencing.

Step 7.

Preparative PCRs were carried out in 25 µl volumes:

ITS PCR Master Mix (1 reaction)

12.5µl	Phusion High-Fidelity PCR Master Mix with HF Buffer (F-531L)
2.5µl	forward primers: ITS1-F_KYO2 (ITS1) ITS86R (ITS2)
2.5µl	reverse primers: ITS86F (ITS1) ITS4 (ITS2)
5.0µl	nuclease-free water
2.5µl	template DNA (10ng/µl)
25µl	**total reaction volume**

Step 8.

For each purified DNA (three DNA isolations per soil sample), the PCRs were performed at three different annealing temperatures at 56°C ± 2°C. The PCRs with unpurified template DNA were performed twice at 56°C. In summary, 15 PCRs were performed per soil sample and ITS region and all amplicons were generated in a Basic & Gradient Labcycler (Sensoquest, Germany).

ITS PCR conditions

Step 1	94°C	5 min
Step 2	94°C	15 sec
Step 3	56°C ± 2°C	25 sec
Step 4	72°C	20 sec
Step 5	72°C	7 min

The PCR fragments were checked on 2% agarose gels with 100bp marker DNA ladder. The gel was stained with Roti®-Gelstain.

Preparation of sequencing pools

Step 9.

The amplicons originating from the same barcode primers were pooled and the concentrations were measured using Qubit® 3.0 (invitrogen) and dsDNA HS Assay Kit (invitrogen). The measurement based on 1µl of each amplicon pool. Aliquots of the pools were concentrated with Eppendorf Concentrator and mixed with 6x Roti®-Load DNA-orange I Dye (Roth, Germany) to track the samples during gel electrophoresis using low-melt agarose (1.5%) and 150ng GeneRuler 50bp Ladder (Peqlab, Germany) mixed with 1.5µl Midori Green Direct Stain (Nippon Genetics Europe, Germany).

Unstained amplicons were excised from the gel according to migration of the separately stained and visualized DNA size markers (molecular weight range from 200 – 500bp).

NOTES

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Amplicons for sequencing were not stained due to possible negative effects on the following DNA sequencing reaction.

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It is important ensuring the gels run for the same time.

Step 10.

Gel sclices were purified using the MinElute Extraction Kit (Qiagen).

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The purified amplicons were eluted in 12µl 10mM Tris HCl (pH 8.5) instead of 10µl.

Step 11.

The concentrations of the amplicon samples were determined using Qubit® 3.0 (invitrogen) and dsDNA HS Assay Kit (invitrogen) and 1µl of each sample.

Step 12.

The amplicons samples were pooled in equimolar amounts separately for ITS1 and ITS2 and diluted to 10nM with 10mM Tris HCl (pH 8.5) and are ready for Illumina Amplicon sequencing steps (e.g. library construction).