Amplification and Illumina Sequencing of ITS1F and ITS4 region of ITS

Introduction

The protocol detailed here is for designed to amplify the ITS1F and ITS4 region of ITS for paired-end fungal ITS community sequencing on the Illumina MiSeq platform using.

Primers for amplification of ITS1F and ITS4 region of ITS

ILM_ITS1F_Uv2 – forward primer

Field number (space-delimited), description:

1. 5' Illumina adapter
2. Forward primer pad
3. Forward primer linker
4. Forward primer (1391f)

AATGATACGGCGACCACCGAGATCTACAC TGTCCGGCTT CG ***CTTGGTCATTTAGAGGAAGTAA***

ILM_ITS4Rv2_00NN – reverse primer

1. Reverse complement of 3' Illumina adapter
2. Golay barcode*
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (EukBr)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCCGTCCG GA ***TCCTCCGCTTATTGATATGC***

*Golay barcodes described by Caporaso et al.

Preparation of master mix for amplification of ITS1F and ITS4 region of ITS

Complete reagent recipe (master mix) for 1X PCR reaction

Component	Volume 1 rxn	Final Conc.
10x ImmoBuffer [(a)]	2.5	1 x
10 mM dNTP	0.5	200 nM
50mM MgCl2	1.25	2.5 mM
ILM_ITS1F_Uv2 (5µM)	2.5	500 nM
ILM_ITS4Rv2 _XXXX (5µM)	2.5	500 nM
Immolase DNA Polymerase (5U/µL) [(a)]	0.2	1 Unit

H_2O	14.55	-
Template	1	-
Total Volume	25	-

[(a)] Immolase DNA Polymerase, Bioline Cat # BIO-21047.

Thermocycler Conditions for amplification of ITS1-F and ITS4 region of ITS (96 well thermocyclers)

	Temperature	Time (mm:ss)
Activation	95°C	10:00
Amplification (35 cycles)	94°C	00:30
	55°C	01:00
	94°C	01:00
Final Extension	94°C	10:00

Process

1. Dilute DNA 1:10.
2. Amplify samples with conditions outlined above
3. Clean amplicon using AMpure XP according to the manufacturer protocol (Beckman Item # 5067-1504)
4. Run amplicons on an agarose gel. Expected band size for ITS1-F/ITS4 is approx. 850 bp.
5. Quantify amplicons with Picogreen (see manufacturers protocol; Invitrogen Item # P11496).
6. Run on Agilent Bioanalyzer chip or Screen Tape to get accurate sizing information.
7. Combine equimolar amounts of amplicons into a single, sterile tube.
8. Optional: If spurious bands were present on gel (in step 3), ½ of the final pool can be run on a gel and then gel extracted to select only the target bands.

Measure concentration using Qubit or picogreen of final pool that has been cleaned and check the 260/280 ratio. For best results the 260/280 should be between 1.8-2.0.

Sequencing of ITS region

Sequencing Primers

Read 1 Primer (ILM_ITS_R1v2)

ACACTGTCCGGCTTCGCTTGGTCATTTAGAGGAAGTAA

Read 2 Primer (ILM_ITS_R2v2)

AGTCCGTCCGGATCCTCCGCTTATTGATATGC

Index Read Primer (ILM_ITS_INDEXv3)

GCATATCAATAAGCGGAGGATCCGGACGGACT

Process

1. Dilute pool prepared in **step 7 above** to **4nM**.
2. Denature according to Ilumina protocol
3. Modify sample sheet to include custom primer's sequence/indexes (see index sequences in appendix 2)
4. Add custom sequencing primers into reservoirs 12-14.

References

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J

Gardes, M., and T. D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113-118

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