# Amplification and Illumina Sequencing of ITS1F and ITS4 region of ITS

## 1.0 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.

## 2.0 Amplification of ITS1F and ITS4 region of ITS

### 2.1 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 (ITS1F)

AATGATACGGCGACCACCGAGATCTACAC TGTCCGGCTT CG CTTGGTCATTTAGAGGAAGTAA

**ILM_ITS4Rv2_00NN – Reverse primer**
Each sequence contains different 12 base Golay barcode as described by Caporaso et al.
1. Reverse complement of 3' Illumina adapter
2. Golay barcode
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (ITS4)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCCGTCCG GA TCCTCCGCTTATTGATATGC

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

| Component | Volume (uL) 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, #BIO-21047)

## 2.3 Thermocycler Conditions for amplification of ITS1F and ITS4 region of ITS (96 well thermocyclers)

| | Temperature | Time (mm:ss) |
|---|---|---|
| Activation | 94°C | 10:00 |
| Amplification (35 cycles) | 94°C | 00:30 |
| | 55°C | 01:00 |
| | 72°C | 01:00 |
| Final Extension | 72°C | 10:00 |
| HOLD | 4°C | ∞ |

## 2.4 Process

2.4.1 Optional: Dilute DNA 1:10.

2.4.2 Amplify samples with conditions outlined above.

2.4.3 Run amplicons on an agarose gel. Expected band size for 1391f/Eukbr is approximately 850 bp.

2.4.4 If there is no band present, repeat PCR using either the undiluted DNA or a 1:100 dilution. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used neat.

2.4.5 Clean and normalize the PCR products. For this step, both Agencourt AMPure XP bead-clean-up and SequalPrep Normalization plates (Invitrogen, A1051001) are acceptable.

*If using Agencourt AMPure XP beads:*

a. Perform a bead clean-up following manufacturer's instructions.
b. Quantify amplicon yields. Acceptable methods include Picogreen (see manufacturers protocol; Invitrogen Item #P11496) or the high sensitivity Quant-iT™ DNA Assay Kit (Life Technologies, Q-33120).
c. Run clean amplicons on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
d. Normalise amplicons by diluting to 10nM with variable volume of buffer (EB buffer, or 10mM Tris pH 8.5) and a set volume of the amplicons.
e. Pool equal volumes of each normalized amplicon.

*If using SequalPrep Normalization plates:*

f. Perform a plate normalization following manufacturer's instructions.
g. Pool equal volumes of each normalized amplicon.
h. Run clean amplicon pool on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.

2.4.6 Optional: If spurious bands were present on gel (in step 2.4.3), or in the clean amplicons (step 2.4.5c) or amplicon pool (step 2.4.5h), a portion of the final amplicon pool can go through a gel extraction or a second round of bead clean up. A lower bead to template ratio will get rid of small bands.

2.4.7 Measure concentration of the final clean pool using Qubit or picogreen.

# 3.0 Sequencing of ITS1F and ITS4 region of ITS

## 3.1 Sequencing Primers

**Read 1 Primer (ILM_ITS_R1v2)**

Field description (space-delimited):
1. Forward primer pad
2. Forward primer linker
3. Forward primer

ACACTGTCCGGCTT CG CTTGGTCATTTAGAGGAAGTAA

**Read 2 Primer (ILM_ITS_R2v2)**

Field description (space-delimited):
1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer

AGTCCGTCCG GA TCCTCCGCTTATTGATATGC

**Index Read Primer (ILM_ITS_INDEXv3)**

Field description (space-delimited):
1. Reverse complement of reverse primer
2. Reverse complement of reverse primer linker
3. Reverse complement of reverse primer pad

GCATATCAATAAGCGGAGGA TC CGGACGGACT

## 3.2 Sequencing Setup

3.2.1 Dilute pool prepared in step 2.4.5 to 4nM.

3.2.2 Denature according to Ilumina protocol, with increased PhiX control spike-in as recommended for low diversity libraries. See *Preparing Libraries for Sequencing on the MiSeq (part #15039740)*.

*3.2.3* Prepare MiSeq Reagent Cartridge (v3 600-cycles). See *MiSeq Reagent Kit v3 - Reagent Preparation Guide (part # 15044983).*

3.2.4 Using an extra long pipette tip set to 75 µL, add 3.4 µL of Read1 sequencing primer (100 µM) into well 12 of the MiSeq Reagent Cartridge and mix 10 times. Repeat adding the Index Primer into well 13 and the Read2 sequencing primer into well 14.

3.2.5 Load 600 µL of library pool into the MiSeq reagent cartridge in designated sample well.

3.2.6 Modify sample sheet to include the custom index sequences.

3.2.7 Start sequencing run following *MiSeq System User Guide (part # 15027617).*

## 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

White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 In: PCR Protocols: A Guide to Methods and Applications, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York.