EMP ITS Illumina Amplicon Protocol

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Abstract

The ITS protocol detailed here is designed to amplify fungal microbial eukaryotic lineages using paired-end community sequencing on the Illumina platform with primers ITS1f-ITS2 (EMP.ITSkabir).

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Guidelines

Ordering primers

The primer sequences in this protocol are always listed in the 5′ -> 3′ orientation. This is the orientation that should be used for ordering. See the page Primer Ordering and Resuspension for more information. Primer constructs were designed by Dylan Smith and Kabir Peay.

- Illumina ITS Primer Constructs (ITS1f-ITS2)

Note: Unlike the 16S and 18S sequencing primers, the ITS sequencing primers have additional 3′ bases beyond the PCR primers, in order to match the melting temperature of the Illumina adapters. The forward sequencing primer has 19 and the reverse sequencing primer has 15 additional 3′ bases; therefore the amplicon sequences will begin 19 bp (forward read) and 15 bp (reverse read) after the PCR primers.

EMP.ITSkabir forward primer (ITS1f)

Field descriptions (space-delimited):

1. 5′ Illumina adapter
2. Forward primer linker
3. Forward primer (ITS1f; Note: This is 38 bp upstream of ITS1 from White et al., 1990.)

AATGATACGGCGACCACCGAGATCTACAC GG CTTGGTCATTTAGAGGAAGTAA

EMP.ITSkabir reverse primer (ITS2), barcoded

Field descriptions (space-delimited):

1. Reverse complement of 3′ Illumina adapter
2. Golay barcode
3. Reverse primer linker
4. Reverse primer (ITS2; Note: This is identical to ITS2 from White et al., 1990.)

CAAGCAGAAGACGGCATACGAGAT NNNNNNNNNN CG GCTGCGTTCTTCATCGATGC

PCR reaction mixtures

Reagent	Volume
PCR-grade water	13.0 µL
PCR master mix (2x)	10.0 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Template DNA	1.0 µL
Total reaction volume	25.0 µL

Notes:

- PCR-grade water from Sigma (cat. no. W3500) or MoBio (cat. no. 17000-11)
- Platinum Hot Start PCR Master Mix (2x) from ThermoFisher (cat. no. 13000014)
- Final master mix concentration in 1x reaction: 0.8x
- Final primer concentration in 1x reaction: 0.2 µM

Thermocycler conditions

ITS amplification

- Primers: ITS1f-ITS2
- Amplicon size: ~230 bp

Temperature	Time	Repeat
94 °C	1 min	
94 °C	30 s	x35
52 °C	30 s	x35
68 °C	30 s	x35
68 °C	7 min	
4 °C	hold	

References

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Before start

For running these libraries on the MiSeq and HiSeq, please make sure you read the supplementary methods of Caporaso et al. (2012). You will need to make your sample more complex by adding 5-10% PhiX to your run.

Materials

PCR-Grade Water W3500 by Sigma Aldrich

Platinum Hot Start PCR Master Mix (2x) 13000014 by Thermo Fisher Scientific

Quant-iT™ PicoGreen™ dsDNA Assay Kit P11496 by Invitrogen - Thermo Fisher

UltraClean PCR Clean Up Kit 12500-250-1 by Mobio

Protocol

Amplification Protocol

Step 1.

Amplify samples in triplicate.

NOTES

Luke Thompson 07 Apr 2018

Each sample will be amplified in 3 replicate 25-µL PCR reactions.

Amplification Protocol

Step 2.

Pool triplicate PCR reactions for each sample into a single volume (75 µL). Do not combine amplicons from different samples at this point.

Amplification Protocol

Step 3.

Run amplicons from each sample on an agarose gel.

EXPECTED RESULTS

Expected band size for ITS1f-ITS2 is ~230 bp.

NOTES

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Low-biomass samples may yield faint or no visible bands; alternative methods such as a Bioanalyzer could be used to verify presence of PCR product.

Amplification Protocol

Step 4.

Quantify amplicons with Quant-iT PicoGreen dsDNA Assay Kit.

REAGENTS

Quant-it™ PicoGreen® dsDNA Assay Kit P7589 by Life Technologies

NOTES

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Follow manufacturer's instructions.

Amplification Protocol

Step 5.

Combine an equal amount of amplicon from each sample (240 ng) into a single, sterile tube. Higher amounts can be used if the final pool will be gel-isolated or when working with low-biomass samples.

NOTES

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When working with multiple plates of samples, it is typical to produce a single tube of amplicons for each plate of samples.

Amplification Protocol

Step 6.

Clean amplicon pool using MoBio UltraClean PCR Clean-Up Kit.

REAGENTS

UltraClean PCR Clean Up Kit 12500-250-1 by Mobio

NOTES

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If working with more than 96 samples, the pool may need to be split evenly for cleaning and then recombined.

If spurious bands were present on gel (in step 3), one-half of the final pool can be run on a gel and then gel extracted to select only the target bands.

Amplification Protocol

Step 7.

Measure concentration and A260/A280 ratio of final pool that has been cleaned.

EXPECTED RESULTS

For best results the A260/A280 ratio should be between 1.8-2.0.

Amplification Protocol

Step 8.

Send an aliquot for sequencing along with sequencing primers listed below.

ITS sequencing primers

Read 1 sequencing primer

Field descriptions (space-delimited):

1. Forward primer segment
2. Extended region into amplicon

TTGGTCATTTAGAGGAAGTAA AAGTCGTAACAAGGTTTCC

Read 2 sequencing primer

Field descriptions (space-delimited):

1. Reverse primer segment
2. Extended region into amplicon

CGTTCTTCATCGATGC VAGARCCAAGAGATC

Index sequencing primer

1. Reverse complement of extended amplicon region
2. Reverse complement of reverse primer
3. Reverse complement of linker

TCTC GCATCGATGAAGAACGCAGC CG

Warnings

Please refer to the SDS (Safety Data Sheet) for safety and hazard information.