

Fungal Metagenomic Sequencing Demonstrated Protocol

Preparing ITS Amplicons for Sequencing on Illumina Sequencing Systems

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Introduction

The Fungal Metagenomic Sequencing Demonstrated Protocol protocol is for the analysis of fungal or metagenomic samples, and describes primer sequences and an assay workflow for use with a BaseSpace Sequence Hub analysis solution. The Internal Transcribed Spacer (ITS) of the rRNA cistron is a useful DNA barcode for characterization of fungal and diverse environmental samples such as stool, sputum, skin, soil, and water. There is ongoing debate on which ITS region is best for fungal genetic analysis (ITS1/ITS2), but both are used widely for metagenomics studies. This multiplex PCR (mPCR) protocol details a workflow for the amplification and analysis of the ITS1 region to provide accurate classification resolution for a broad range of taxa.

Workflow Summary

- 1 Order amplicon primers—Some primers for the protocol must be ordered from a third-party oligonucleotide manufacturer. For more information, see *Amplicon Primers* on page 12.
- 2 Prepare library—Amplify the ITS1 region using a limited cycle PCR, and then add Illumina sequencing adapters and dual-index barcodes to the amplicon target. Up to 384 libraries can be pooled together for sequencing with Nextera™ XT v2 indexes. Alternatively, using Nextera DNA CD Indexes, up to 96 libraries can be pooled together for sequencing.
- 3 Sequence—Sequencing with 2x150 cycles and 15,000–100,000 reads per sample provides high classification resolution and accuracy. Multiplexing capability depends on the sequencing platform used.
- 4 Analyze with the BaseSpace ITS Metagenomics App—The ITS Metagenomics Workflow performs a taxonomic classification using the UNITE database¹ showing classification across all taxonomic levels in a graphical format.



NOTE

This protocol can be used to sequence ITS2 by changing the locus-specific sequence of the amplicon primers. For more information, see *Amplicon Primers* on page 12.



DISCLAIMER

The information in this Illumina Demonstrated Protocol is being provided as a courtesy. In some cases, reagents are required to be purchased from nonauthorized third-party suppliers. Illumina does not guarantee or promise technical support for the performance of our products used with any reagent purchased from a nonauthorized third-party supplier.

Resources

1. Kõljalg U, Nilsson RH, Abarenkov K, et al. Towards a unified paradigm for sequence-based identification of Fungi. *Mol Ecol*. 2013;22(21):5271-5277.

Tips and Techniques

When preparing libraries for sequencing, always adhere to good molecular biology practices. Read through the entire protocol before starting to make sure that all required materials are available and equipment is programmed and ready to use.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- ▶ Small differences in volumes ($\pm 0.5 \mu\text{l}$) can sometimes cause large differences in cluster numbers ($\sim 100,000$).

- ▶ Small volume pipetting can be a source of potential error in protocols requiring the generation of standard curves or small but precise volumes.
- ▶ If small volumes are unavoidable, use due diligence to make sure that pipettes are correctly calibrated.
- ▶ Make sure that pipettes are not used at the volume extremes of their performance specifications.
- ▶ Prepare the reagents for multiple samples simultaneously to minimize pipetting errors, especially with small volume enzyme additions. As a result, pipette one time from the reagent tubes with a larger volume, rather than many times with small volumes. Aliquot to individual samples in a single pipetting movement to allow for standardization across multiple samples.

Handling Beads

- ▶ Before use, allow the beads to come to room temperature.
- ▶ Do not reuse beads. Always add fresh beads when performing these procedures.
- ▶ Immediately before use, vortex the beads until they are well dispersed and the color of the liquid is homogeneous.
- ▶ When pipetting the beads, pipette slowly and dispense slowly due to the viscosity of the solution.
- ▶ Take care to minimize bead loss, which can affect final yields.
- ▶ Change the tips for each sample, unless specified otherwise.
- ▶ Let the mixed samples incubate at room temperature for the time indicated in the protocol for maximum recovery.
- ▶ When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- ▶ When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- ▶ To prevent the carryover of beads after elution, approximately 2.5 μl of supernatant is left when the eluates are removed from the bead pellet.
- ▶ Be sure to remove all the ethanol from the bottom of the wells, as it can contain residual contaminants.
- ▶ Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, because the presence of ethanol affects the performance of the subsequent reactions. Dry for at least 10 minutes, but a longer drying time can be required. Remaining ethanol can be removed with a 10 μl pipette.
- ▶ Avoid overdrying the beads, which can impact final yields.
- ▶ Do not scrape the beads from the edge of the well using the pipette tip.
- ▶ To maximize sample recovery during elution, incubate the sample/bead mix for two minutes at room temperature before placing the samples onto the magnet.



NOTE

Cleanup procedures have only been tested using the 96-well plates and the magnetic stand specified in *Consumables and Equipment* on page 13. Comparable performance is not guaranteed when using a microcentrifuge tube, other formats, or other magnets.

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

- ▶ Open only one adapter tube at a time.
- ▶ When adding or transferring samples, change tips between **each sample** unless instructed otherwise.
- ▶ Pipette carefully to avoid spillage.

- ▶ Clean pipettes and change gloves between handling different adapter stocks.

Temperature Considerations

- ▶ Keep libraries at temperatures $\leq 37^{\circ}\text{C}$, except where specifically noted.
- ▶ Place reagents on ice after thawing at room temperature.

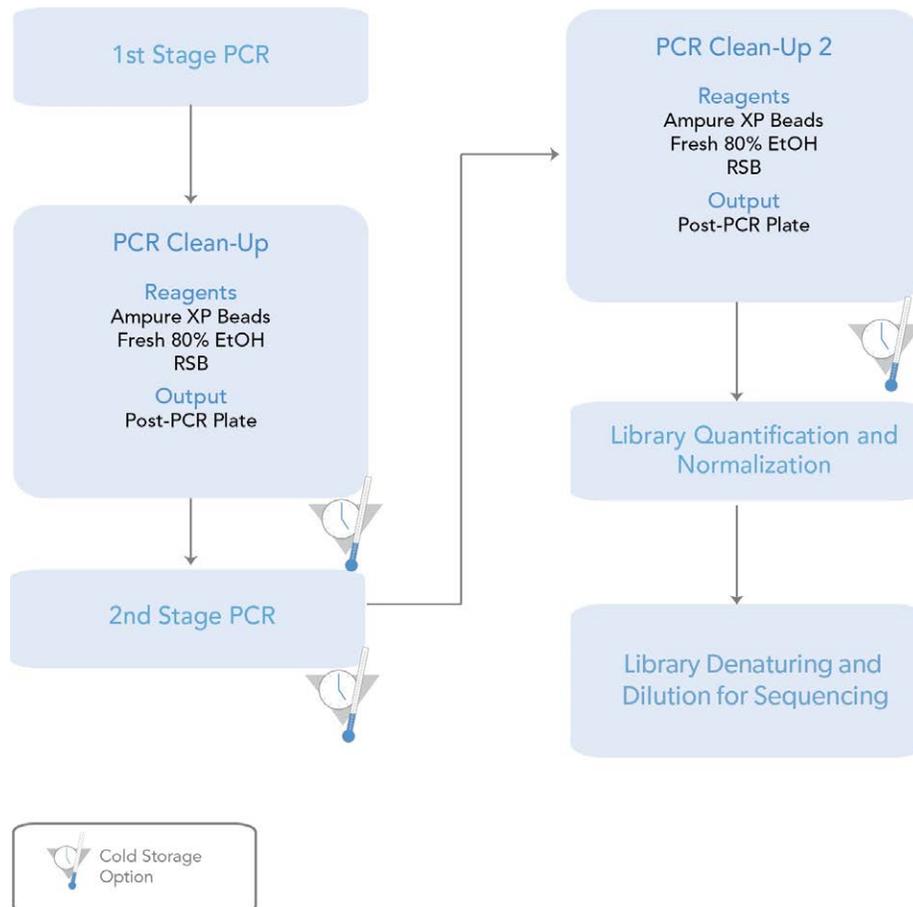
Equipment

- ▶ Review the programming instructions for your thermal cycler user guide to make sure that it is programmed appropriately using the heated lid function.
- ▶ It is acceptable to use the thermal cycler tracked heating lid function.

Library Prep Workflow

The following diagram illustrates the workflow using the ITS Library Preparation Protocol. Safe stopping points are marked between steps.

Figure 1 ITS Library Prep Workflow



Amplicon PCR

This step uses PCR to amplify template out of a DNA sample using region of interest-specific primers with overhang adapters attached. For more information on primer sequences, see *Amplicon Primers* on page 12.



NOTE

For more information on consumables and equipment, see *Consumables and Equipment* on page 13.

Consumables

Item	Quantity	Storage
Microbial Genomic DNA (5 ng/μl in 10 mM Tris pH 8.5)	2.5 μl per sample	-25°C to -15°C
Amplicon PCR reverse primer (1 μM)	5 μl per sample	-25°C to -15°C
Amplicon PCR forward primer (1 μM)	5 μl per sample	-25°C to -15°C
2x KAPA HiFi HotStart ReadyMix	12.5 μl per sample	-25°C to -15°C
Microseal 'A' film		
96-well 0.2 ml PCR plate	1 plate	
[Optional] Bioanalyzer chip (Agilent DNA 1000 kit catalog # 5067-1504)		

Procedure

- 1 Add the following volumes to each sample well :

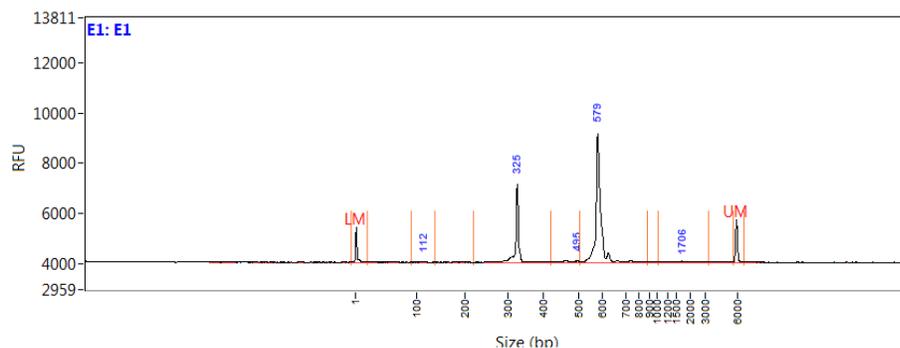
Reagent	Volume (μl)
Microbial DNA (5 ng/μl)*	2.5
Amplicon PCR forward primer or primer pool (1 μM)**	5
Amplicon PCR reverse primer or primer pool (1 μM)**	5
2x KAPA HiFi HotStart ReadyMix	12.5
Total	25

* Assay can perform well with total DNA input as low as 0.1 ng with high-quality fungal gDNA. Because fungal content in environmental samples can vary, increased input is recommended.

** If using recommended primers or other sets of primers, dilute aliquot of each primer to 1 μM with 1X TE pH 8 buffer. Pool equal volumes of each primer to create a forward and reverse primer pool. Aliquot 5 μl of each primer pool into each library plate well containing sample.

- 2 Seal plate and perform PCR on a thermal cycler using the following program:
 - ▶ 95°C for 3 minutes
 - ▶ 25 cycles of:
 - ▶ 95°C for 30 seconds
 - ▶ 55°C for 30 seconds
 - ▶ 72°C for 30 seconds
 - ▶ 72°C for 5 minutes
 - ▶ Hold at 4°C
- 3 [Optional] Run 1 μl of the PCR product on your fragment analyzer of choice (eg, Agilent Bioanalyzer DNA1000 or AATI Fragment Analyzer High Sensitivity Kit diluted 1:10) to verify successful amplification. Typical libraries show a size distribution from ~145–695 bp.
The ITS1 amplicon varies in length relative to taxa.

Figure 2 Example Fragment Analyzer Trace After Amplicon PCR Step



PCR Cleanup

This step uses AMPure XP beads to purify the ITS1 amplicon away from free primers and primer dimer species.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	52.5 µl per sample	-25°C to -15°C
AMPure XP beads	20 µl per sample	2°C to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well midi plate	1 plate	

Preparation

- 1 Bring the AMPure XP beads to room temperature.

Procedure

- 1 Centrifuge the amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, and then carefully remove seal.
- 2 [Optional] If planning to use a shaker for mixing, transfer the entire amplicon PCR product from the PCR plate to the midi plate.
- 3 Vortex the AMPure XP beads for 30 seconds to disperse beads evenly.
- 4 Add an appropriate volume of beads to a trough depending on the number of samples.
- 5 Add 20 µl of AMPure XP beads to each well of the amplicon PCR plate.
- 6 Select from one of the following options:
 - ▶ 96-well PCR plate—Pipette entire volume up and down 10 times.
 - ▶ Midi plate—Seal plate and shake at 1800 rpm for 2 minutes.
- 7 Incubate at room temperature for 5 minutes.
- 8 Place the plate on the magnetic stand and wait until the supernatant is clear (~2 minutes). Keep on the magnetic stand until step 13.

- 9 Remove and discard the supernatant.
- 10 Wash beads two times as follows.
 - a Add 200 μ l of 80% ethanol to each sample well.
 - b Incubate the plate on the magnetic stand at room temperature for 30 seconds.
 - c Remove and discard the supernatant.
- 11 Remove excess ethanol.
- 12 Air-dry for 10 minutes.
- 13 Add 52.5 μ l of 10 mM Tris pH 8.5 to each well.
- 14 Select from one of the following options:
 - ▶ 96-well PCR plate—Pipette entire volume up and down 10 times.
 - ▶ Midi plate—Seal plate and shake at 1800 rpm for 2 minutes.
Make sure that beads are fully resuspended.
- 15 Incubate at room temperature for 2 minutes.
- 16 Place the plate on the magnetic stand and wait until the supernatant is clear (~2 minutes).
- 17 Carefully transfer 50 μ l of the supernatant from the amplicon PCR plate to a new 96-well PCR plate.

SAFE STOPPING POINT

If you do not immediately proceed to *Index PCR* on page 7, seal plate with Microseal 'B' adhesive seal and store at -15°C to -25°C for up to a week.

Index PCR

This step attaches dual indexes and Illumina sequencing adapters using the Nextera XT Index Kit v2, Nextera DNA CD Indexes (24 Indexes, 24 Samples) (tubes), or Nextera DNA CD Indexes (96 Indexes, 96 Samples) (plate).

Consumables

Item	Quantity	Storage
2x KAPA HiFi HotStart ReadyMix	25 μ l per sample	-25°C to -15°C
Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index Kit v2 set A, B, C, or D (FC-131-2001, FC-131-2002, FC-131-2003, or FC-131-2004)	5 μ l per sample	-25°C to -15°C
Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index Kit v2 set A, B, C, or D (FC-131-2001, FC-131-2002, FC-131-2003, or FC-131-2004)	5 μ l per sample	-25°C to -15°C
Nextera DNA CD Index 1 Primers from the Nextera DNA CD Index Kit (24 Indexes, 24 Samples) (20018707)	5 μ l per sample	-25°C to -15°C
Nextera DNA CD Index 2 Primers from the Nextera DNA CD Index Kit (24 Indexes, 24 Samples) (20018707)	5 μ l per sample	-25°C to -15°C
Nextera DNA CD Index 1 and 2 Primer Mix from the Nextera DNA CD Index Kit (96 Indexes, 96 Samples) (20018708)	10 μ l per sample	-25°C to -15°C
Water, PCR-grade	10 μ l per sample	-25°C to -15°C

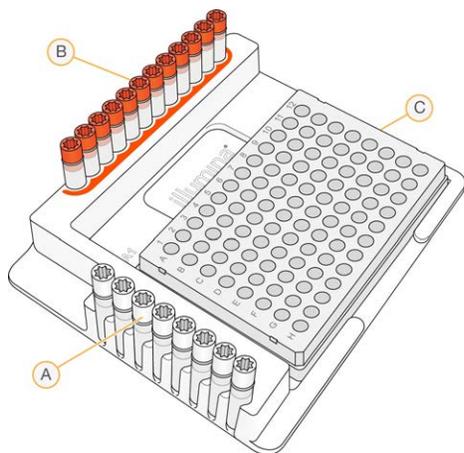
Item	Quantity	Storage
TruSeq Index Plate Fixture (FC-130-1005)	1	
96-well 0.2 ml PCR plate	1 plate	
Microseal 'A' film	1	

Procedure

- Transfer 5 μ l of library from each well to a new 96-well plate.
The remaining 45 μ l is not used in the protocol and can be stored for other uses.
- Arrange the Index 1 and 2 primers in a rack (eg, the TruSeq Index Plate Fixture) using the following arrangements as needed:
 - Arrange Index 2 primer tubes (white caps) vertically, aligned with rows A through H.
 - Arrange Index 1 primer tubes (orange caps) horizontally, aligned with columns 1 through 12.

For more information on index selection, see *Dual-Indexing Principle* on page 15.

Figure 3 TruSeq Index Plate Fixture



- A Index 2 primers (white caps)
- B Index 1 primers (orange caps)
- C 96-well plate

- Place the 96-well PCR plate with the 5 μ l of resuspended PCR product DNA in the rack.
- Add the following volumes, as appropriate for the index kit in use, to each sample well :

Reagent	Volume (μ l)
Post-Cleanup PCR1 Library	5
Nextera XT Index Primer 1 (N7xx)	5
Nextera XT Index Primer 2 (S5xx)	5
CDIndex Primer 1 (24)	5
CDIndex Primer 2 (24)	5
CDIndex Primer 1, 2 Mix (96)	10
2x KAPA HiFi HotStart ReadyMix	25

Reagent	Volume (µl)
Water, PCR-grade	10
Total	50

- 5 Pipette up and down 10 times to mix.
- 6 Cover the plate with Microseal 'A'.
- 7 Centrifuge the plate at 1,000 × g at 20°C for 1 minute
- 8 Perform PCR on a thermal cycler using the following program:
 - ▶ 95°C for 3 minutes
 - ▶ Eight cycles of:
 - ▶ 95°C for 30 seconds
 - ▶ 55°C for 30 seconds
 - ▶ 72°C for 30 seconds
 - ▶ 72°C for 5 minutes
 - ▶ Hold at 4°C

Second PCR Cleanup

This step uses AMPure XP beads to clean up the final library before quantification.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	27.5 µl per sample	-25°C to -15°C
AMPure XP beads	56 µl per sample	2°C to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well midi plate	1 plate	

Procedure

- 1 Centrifuge the index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
- 2 [Optional] If planning to use a shaker for mixing, transfer the entire amplicon PCR product from the PCR plate to the midi plate.



NOTE

Transfer the sample to a 96-well midi plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.

- 3 Vortex the AMPure XP beads for 30 seconds to disperse beads evenly.
- 4 Add an appropriate volume of beads to a trough.
- 5 Add 56 µl of AMPure XP beads to each well of the index PCR plate.
- 6 Select from one of the following options:
 - ▶ 96-well PCR plate—Pipette entire volume up and down 10 times.
 - ▶ Midi plate—Seal plate and shake at 1800 rpm for 2 minutes.

- 7 Incubate at room temperature for 5 minutes.
- 8 Place the plate on the magnetic stand and wait until the supernatant is clear (~2 minutes). Keep on the magnetic stand until step 13.
- 9 Remove and discard the supernatant.
- 10 Wash beads two times as follows.
 - a Add 200 μ l of 80% ethanol to each sample well.
 - b Incubate the plate on the magnetic stand at room temperature for 30 seconds.
 - c Remove and discard the supernatant.
- 11 Remove excess ethanol.
- 12 Air-dry for 10 minutes.
- 13 Add 27.5 μ l of 10 mM Tris pH 8.5 to each well.
- 14 Select from one of the following options:
 - ▶ 96-well PCR plate—Pipette entire volume up and down 10 times.
 - ▶ Midi plate—Seal plate and shake at 1800 rpm for 2 minutes. Make sure that beads are fully resuspended.
- 15 Incubate at room temperature for 2 minutes.
- 16 Place the plate on the magnetic stand and wait until the supernatant is clear (~2 minutes).
- 17 Carefully transfer 25 μ l of the supernatant from the index PCR plate to a new 96-well PCR plate.

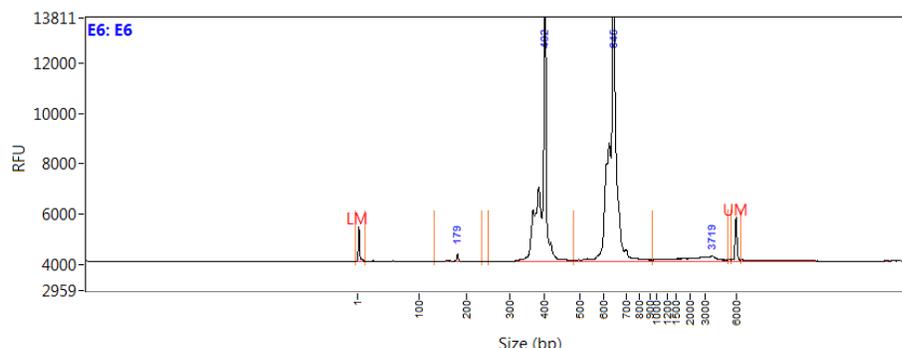
SAFE STOPPING POINT

If you do not plan to proceed to Library Quantification, Normalization, and Pooling, seal plate with Microseal 'B' adhesive seal and store at -15°C to -25°C for up to a week.

[Optional] Check Library

- 1 Validate the library using one of the following methods:
 - ▶ Analyze 1 μ l of a 1:50 dilution on a Bioanalyzer DNA 1000 chip.
 - ▶ Analyze 1 μ l of a 1:20 dilution on an AATI High Sensitivity Kit.
 Typical libraries show a size distribution from ~225–775 bp.
 The ITS1 amplicon varies in length relative to taxa.

Figure 4 Example Bioanalyzer Trace of Final Library



Library Quantification, Normalization, and Pooling

- 1 Quantify the library using a fluorometric quantification method that uses dsDNA binding dyes.
- 2 Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace:

$$\frac{\text{ng} / \mu\text{l} \times 10^6}{660 \frac{\text{g}}{\text{mol}} \times \text{average library size (bp)}} = \text{Molarity}$$

For example:

$$\frac{15 \text{ ng} / \mu\text{l} \times 10^6}{660 \frac{\text{g}}{\text{mol}} \times 500} = 45 \text{ nM}$$

- 3 Dilute concentrated final library using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 to 4 nM.
- 4 Aliquot 5 μl of diluted DNA from each library and mix aliquots for pooling libraries with unique indexes. For metagenomics samples, >15,000–100,000 reads per sample is sufficient to fully survey the fungal composition. This number of reads allows for sample pooling depending on sequencing platform and sequencing kit, up to 384 samples per run.

Sample Loading

- 1 Denature and dilute the library pool to the appropriate loading concentration for the sequencing run. See the denature and dilute libraries instructions for your sequencing system.

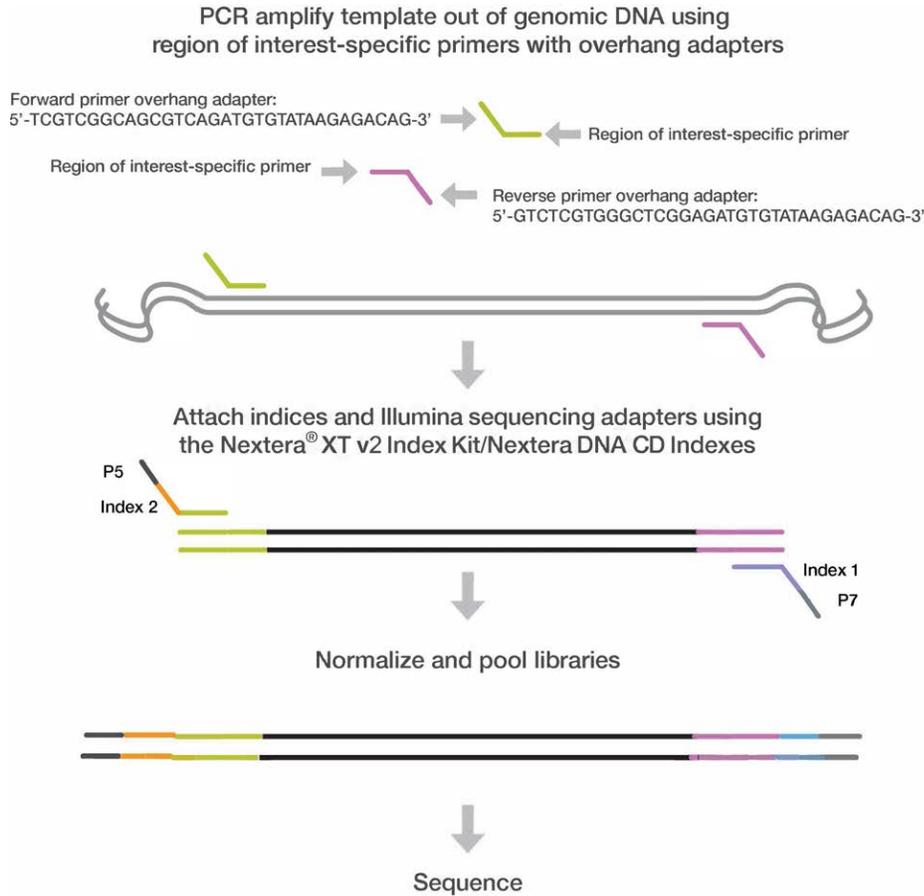
Supporting Information

The protocols described in this guide assume that you are familiar with the contents of this section and have obtained all of the requisite equipment and consumables.

ITS Amplicon Workflow

User-defined forward and reverse primers that are complementary upstream and downstream of the region of interest are designed with overhang adapters. These primers are then used to amplify templates from genomic DNA. A subsequent limited-cycle amplification step is performed to add multiplexing indexes and Illumina sequencing adapters. Libraries are then normalized and pooled before sequencing.

Figure 5 ITS Amplicon Workflow



Amplicon Primers

The gene-specific sequences used in this protocol target the fungal ITS1 region between the 18S and 5.8S rRNA genes. They include the ITS1-F and ITS2 primers from Bellemain et al.,¹ which are widely used for fungal barcoding studies.

To assess potential performance of these primer sequences, we performed probe hit analyses. Because the ITS1 sequence was truncated in our analysis database (RDP Classifier Fungal ITS Warcup Training Set V2²) and did not contain the forward primer, separate probe hit analyses were performed for the forward and reverse primer sequences. When carrying out probe hit analysis on the original reverse primer sequence against the Warcup ITS training set, gaps were found in taxonomic coverage. Additional primers were added to the reverse primer pool to address these gaps. Similar analyses were done using the SILVA database for the forward primer sequence to identify primer sequences to add to the forward primer pool. The primer sequences for the ITS1 region are listed in Table 1 and Table 2.

Table 1 Forward Primer Set

Name	Sequence (5')
ITS_fwd_1	CTTGGTCATTTAGAGGAAGTAA
ITS_fwd_2	CTCGGTCATTTAGAGGAAGTAA
ITS_fwd_3	CTTGGTCATTTAGAGGAAGTAA
ITS_fwd_4	CCCGGTCATTTAGAGGAAGTAA

Name	Sequence (5')
ITS_fwd_5	CTAGGCTATTTAGAGGAAGTAA
ITS_fwd_6	CTTAGTTATTTAGAGGAAGTAA
ITS_fwd_7	CTACGTCATTTAGAGGAAGTAA
ITS_fwd_8	CTTGGTCATTTAGAGGTCGTAA

Table 2 Reverse Primer Set

Name	Sequence (5')
ITS_rev_1	GCTGCGTTC TTCATCGATGC
ITS_rev_2	GCTGCGTTC TTCATCGATGG
ITS_rev_3	GCTACGTTCTTCATCGATGC
ITS_rev_4	GCTGCGTTC TTCATCGATGT
ITS_rev_5	ACTGTGTTCTTCATCGATGT
ITS_rev_6	GCTGCGTTC TTCATCGTTGC
ITS_rev_7	GCGTTCTTCATCGATGC

Gene-specific sequences can also be utilized to target other regions on the genome (either for ITS1 with other sets of primer pairs, or non-ITS1 regions throughout the genome like ITS2 or D1/D2). The overhang adapter sequence must be added to the locus-specific primer for the region to be targeted (see Figure 5). The Illumina overhang adapter sequences to be added to locus-specific sequences are listed in Table 3.

Table 3 Overhang Adapter Sequences

Name	Sequence (5')
Forward overhang: 5'	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]
Reverse overhang: 5'	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]

The following considerations are recommended for designing other locus-specific primers:

- ▶ The locus-specific portion of primer (not including overhang sequence) must have a melting temperature (T_m) compatible with PCR annealing temperature. Use online PCR primer sequence analysis tools to check the properties of primer designs. For the T_m calculation only, the gene-specific portion must be used. For hairpin and dimer calculations, the fully assembled primer sequence (including the overhang) should be used.
- ▶ Use standard desalting purification when ordering oligo primer sets.



NOTE

For more information on reagents used in the protocol, see *Consumables and Equipment* on page 13.

Resources

1. Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. *BMC Microbiol.* 2010;10(189).
2. Deshpande V, Wang Q, Greenfield P, et al. Fungal identification using a Bayesian classifier and the Warcup training set of internal transcribed spacer sequences. *Mycologia.* 2015;108(1):1-5.

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

Table 4 User-Supplied Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
96-well 0.2 ml skirtless PCR plates or Twin.Tec 96-well PCR plates	Bio-Rad, part # MSP-9601
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Amplicon PCR forward primer (standard desalting)	
Amplicon PCR reverse primer (standard desalting)	
KAPA HiFi HotStart ReadyMix (2X)	KAPA Biosystems, part # KK2601
Microseal 'A' adhesive seals	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MiSeq Reagent Kit v3 (600 cycle)	Illumina, catalog # MS-102-3003
Nextera XT Index Kit v2	Illumina, catalog # FC-131-2001 or Illumina, catalog # FC-131-2002 or Illumina, catalog # FC-131-2003 or Illumina, catalog # FC-131-2004
Nextera DNA CD Indexes	Illumina, catalog # 20018707 or Illumina, catalog # 20018708
PhiX Control Kit v3	Illumina, catalog # FC-110-3001
Water, PCR-grade	General lab supplier
Fluorometric quantitation with dsDNA binding dye reagents	General lab supplier
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
[Optional] 96-well storage plates, round well, 0.8 ml ("midi" plate)	Thermo Scientific, part # AB-0859

Table 5 User-Supplied Equipment

Consumable	Supplier
2.5 L ice bucket	General lab supplier
96-well thermal cycler (with heated lid)	General lab supplier
Fluorometer for quantitation with dsDNA binding dyes	General lab supplier
Magnetic stand-96	Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
TruSeq Index Plate Fixture Kit (reusable)	Illumina, catalog # FC-130-1005
Vortexer	General lab supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Agilent DNA 1000 Kit	Agilent, part # 5067-1504
[Optional] High-Speed Microplate Shaker	VWR, catalog # 13500-890 (110V/120V) or VWR, catalog # 14216-214 (230V)

Dual-Indexing Principle

The dual-indexing strategy adds two 8-base indexes to each sample, Index 1 (i7) adjacent to the P7 sequence and Index 2 (i5) adjacent to the P5 sequence. The Nextera XT Index Kit v2 (FC-131-2001, FC-131-2002, FC-131-2003, or FC-131-2004) uses 24 different Index 1 (i7) adapters and 16 different Index 2 (i5) adapters. In the index adapter name:

- ▶ N or S refers to Nextera XT
- ▶ 7 refers to Index 1 (i7)
- ▶ 5 refers to Index 2 (i5)
- ▶ 01–12 refers to the index number

Use the following index sequences to generate sample sheets to demultiplex the samples:

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	S502	CTCTCTAT
N702	CGTACTAG	S503	TATCCTCT
N703	AGGCAGAA	S505	GTAAGGAG
N704	TCCTGAGC	S506	ACTGCATA
N705	GGACTCCT	S507	AAGGAGTA
N706	TAGGCATG	S508	CTAAGCCT
N707	CTCTCTAC	S510	CGTCTAAT
N710	CGAGGCTG	S511	TCTCTCCG
N711	AAGAGGCA	S513	TCGACTAG
N712	GTAGAGGA	S515	TTCTAGCT
N714	GCTCATGA	S516	CCTAGAGT
N715	ATCTCAGG	S517	GCGTAAGA
N716	ACTCGCTA	S518	CTATTAAG
N718	GGAGCTAC	S520	AAGGCTAT
N719	GCGTAGTA	S521	GAGCCTTA
N720	CGGAGCCT	S522	TTATGCGA
N721	TACGCTGC		

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N722	ATGCGCAG		
N723	TAGCGCTC		
N724	ACTGAGCG		
N726	CCTAAGAC		
N727	CGATCAGT		
N728	TGCAGCTA		
N729	TCGACGTC		

For information on the Nextera DNA CD Index kits, see the *Index Adapters Pooling Guide (document # 1000000041074)* and the Nextera DNA Flex Kit support page.

Low Plexity Pooling Guidelines

Illumina uses a green laser or LED to sequence G/T and a red laser or LED to sequence A/C. At each cycle, at least one of two nucleotides for each color channel are read to ensure proper registration. It is important to maintain color balance for each base of the Index Read being sequenced, otherwise Index Read sequencing could fail due to registration failure. If you choose the dual-indexed sequencing workflow, always use at least two unique and compatible barcodes for each index (index 1 and index 2). The following tables illustrate possible pooling strategies:

Table 6 Pooling Strategies

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
1-plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2-plex	<ul style="list-style-type: none"> • [Option 1] N702 and N701 • [Option 2] N702 and N704 	Any Index 2 adapter
3-plex	<ul style="list-style-type: none"> • [Option 1] N701, N702, and N704 • [Option 2] N703, N705, and N706 	Any Index 2 adapter
4- or 5-plex	<ul style="list-style-type: none"> • [Option 1] N701, N702, N704, and any other Index 1 adapter • [Option 2] N703, N705, N706, and any other Index 1 adapter 	Any Index 2 adapter
6-plex	N701, N702, N703, N704, N705, and N706	Any Index 2 adapter
7–12 plex, Dual Index	<ul style="list-style-type: none"> • [Option 1] N701, N702, N704, and any other Index 1 adapter (as needed) • [Option 2] N703, N705, N706, and any other Index 1 adapter (as needed) 	S505 and S506
7–12 plex, Single Index (96 Sample Nextera Index adapter kit)	N701–N706 and any other Index 1 adapter (as needed)	Any Index 2 (i5) adapter
> 12 plex	N701, N702, N703, N704, N705, N706, and any other Index 1 adapter	S505, S506, and any other Index 2 adapter (as needed)

These strategies represent only some of the acceptable combinations. Alternatively, check the real sequences of each index to make sure that each base position has a signal in both color channels for the Index Read:

Figure 6 Base Position Signals

Good				Bad			
Index 1		Index 2		Index 1		Index 2	
705	GGACTCCT	503	TATCCTCT	705	GGACTCCT	502	CTCTCTAT
706	TAGGCATG	503	TATCCTCT	706	TAGGCATG	502	CTCTCTAT
701	TAAGGCCA	504	AGACTAGA	701	TAAGGCCA	503	TATCCTCT
702	CGTACTAG	504	AGACTAGA	702	CGTACTAG	503	TATCCTCT
	√√√√√√√√		√√√√√√√√		√√√√√√√√		√√√√xxxx

√=signal in both color channels
 x=signal missing in one color channel

Revision History

Document	Date	Description of Change
Document # 1000000064940 v01	February 2019	Corrected sequencing cycle number error. Clarified Amplicon PCR procedure. Added Nextera DNA CD Indexes information.
Document # 1000000064940 v00	September 2018	Initial release.