
iTag Sample Preparation for Illumina Sequencing

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Summary

The purpose of this procedure is to generate Illumina amplicon libraries, iTags, for targeted sequencing of the 16S and 18S ribosomal RNA genes, and the internal transcribed spacer (ITS) regions for fungal species, directly from environmental samples. This procedure requires 30ng of isolated DNA template diluted to 10ng/μl as input material for the sample prep.

Materials & Reagents

- QuantaBio 5PRIME HotMasterMix (VWR Catalog # 10847-706 or 10847-708)
- PCR Grade Water, nuclease-free (VWR Catalog # 10220-396)
- Bovine Serum Albumin (BSA), Molecular Biology Grade (NEB Catalog # B9000S)
- Chloroplast and Mitochondrial PNA blocking oligos for 16S endophyte samples (PNA Bio Catalog # MP01-25 and PP01-25)
- Forward and Reverse PCR Primers for specific iTag application (see **Appendix** section for primer info for ordering):
 - 16S V4 region primers
 - 16S V4-V5 region primers
 - 18S V4 region primers
 - Fungal ITS2 region primers
- Standard lab equipment for performing PCR reactions: Pipettes, pipette filter tips, micro-tubes, 96-well PCR plates, centrifuge, thermal cycler.
- Capability to assess that amplification occurred and to size the amplified product: agarose gel, Bioanalyzer, Fragment Analyzer, or similar method/instrument.
- Capability to quantify the amplified product: Bioanalyzer, Fragment Analyzer, plate reader PicoGreen assay, or similar method/instrument.
- Capability to perform PCR clean-up: MoBio UltraClean PCR Clean-Up Kit (MoBio Catalog # 12500), or AMPure XP beads (Agencourt Catalog # A63880), or similar kit/method to do PCR clean-up.

Health & Safety

Appropriate personal protective equipment should be worn, such as lab coat, gloves, eye protection, long-pants, and closed-toe shoes.

Procedure

NOTE: Sample template input target amount is 30ng at 10ng/μl, which will be divided into three 10ng inputs for triplicate replicate PCR reactions.

NOTE: All reagents/stock solutions should be thawed at 4°C or on wet ice prior to the start of the procedure.

IMPORTANT: Once thawed, mix the 5 PRIME HotMasterMix thoroughly to avoid localized differences in salt concentrations.

1. Prepare the Template & Primer Master-mix

- 1.1. Amplification will be done in triplicate for each sample, meaning each sample will be amplified in three replicate 25μl PCR reactions with 10ng of sample template input for each reaction.
- 1.2. Prepare the template & primer master-mix in PCR tubes or 96-well plates, adding the Template, Forward & Reverse Primers, PCR Grade Water, BSA, 5 PRIME HotMasterMix, and PNA blocking oligos (if using for a 16S endophyte sample) as specified in Table 1:

	PCR without PNA blocking oligos	PCR with PNA blocking oligos
Component	Volume (μl)	Volume (μl)
PCR Grade Water	12.0	11.4
BSA (10mg/ml)	1.0	1.0
5 PRIME HotMasterMix	10.0	10.0
Forward Primer (10μM)	0.5	0.5
Reverse Primer (10μM)	0.5	0.5
Template DNA (10ng/μl)	1.0	1.0
PNA Chloroplast (100 μM)	-	0.3
PNA Mitochondrial (100 μM)	-	0.3
Total 1X reaction volume	25.0	25.0

Table 1: Mix the components at ambient temperature; volumes are for 1X PCR reaction, setup three replicate reactions.

- 1.3. Close the tubes, or seal the plates, and mix well. Centrifuge briefly to collect liquid at the bottom of the tube or plate well.

2. Amplify on Thermal Cycler

- 2.1. Load the PCR tubes or 96-well plate on thermal cyclers and start the PCR program using the cycling conditions in Table 2 for reactions without PNA blocking oligos or Table 3 for reactions with PNA blocking oligos.

NOTE: The thermal cycler should be preheated (>90°C) before placing the PCR tubes or plate on the cycler block.

PCR without PNA blocking oligos		
Step	Temperature	Time
1.	94°C	3 min
2.	94°C	45 sec
3.	50°C	60 sec
4.	72°C	90 sec
5.	Repeat Steps 2-4 for 30 cycles	
6.	72°C	10 min
7.	4°C	HOLD

Table 2: PCR conditions for iTag sample amplification without PNA blocking oligos.

PCR with PNA blocking oligos		
Step	Temperature	Time
1.	94°C	3 min
2.	94°C	45 sec
3.	78°C	10 sec
4.	50°C	60 sec
5.	72°C	90 sec
6.	Repeat Steps 2-5 for 30 cycles	
7.	72°C	10 min
8.	4°C	HOLD

Table 3: PCR conditions for iTag sample amplification with PNA blocking oligos.

3. Assess Amplified Product of each Sample for Quality and Quantity

- 3.1. After amplification, combine the triplicate PCR reactions for each sample into a single volume (75µL); do not combine amplicons from different samples at this point as the individual sample amplicons need to be qualified & quantified.
- 3.2. Run sample amplicon products on an agarose gel or Bioanalyzer (or similar instrument like LabChip or Fragment Analyzer) to check quality of the amplicons and to ensure sample amplification occurred with the expected amplicon band size of (including primers): ~460bp for 16S V4, ~580bp for 16S V4-V5, ~560bp 18S V4, or 370-590bp for ITS2.
 - a. See **Troubleshooting** section for samples that fail to amplify.
- 3.3. Quantify the sample amplicon products using Bioanalyzer, Fragment Analyzer, plate reader PicoGreen assay (or similar method/instrument).

4. Pooling Sample Amplicons and Clean-up for Sequencing

- 4.1. Using the sample amplicon quantification info, combine sample amplicons together in equal amounts by mass or molarity to create a pool for sequencing. Only pool samples together that have unique i7 index sequences on their RV primer.
- 4.2. Clean the pool using a PCR clean-up method, MoBio UltraClean PCR Clean-Up Kit, 1.2X AMPure XP beads, or similar clean-up method/kit.
- 4.3. Quantify the pool using Bioanalyzer, Fragment Analyzer, plate reader PicoGreen assay (or similar method/instrument).

5. Sequencing the Sample Pool

- 5.1. Send an aliquot of the pool for sequencing.
 - a. The prepared amplicons contain Illumina adapter sequences that are compatible with the Illumina sequencing workflow and are compatible with Illumina sequencing primers and index primers; no custom spike-in primers are needed for sequencing.
 - b. Sequencing run mode length should be sufficient to generate overlapping reads that cover the entire amplicon insert.
 - c. Index read 1 (i7) should be sequenced with 12 cycles to sequence the entire 12bp index to be used for demultiplexing the sequence data

Troubleshooting

Assuming no process issues occurred during amplification, then samples that fail to amplify are likely due to the presence of contaminants that are inhibiting the PCR reaction. The following are troubleshooting techniques that can be used to try and overcome inhibition.

1. Dilution of the sample template

- 1.1. Try diluting the sample template and repeating the amplification. This may successfully dilute out the inhibitor and allow for successful amplification. In general, the quality & purity of the sample is more important than the quantity going into the reaction.
- 1.2. Try starting with a 1:10 or greater dilution, or setting up a serial dilution titration series to see at what dilution point the sample begins to amplify.

2. Re-extraction, isolation, or clean-up of the sample template

- 2.1. Many PCR inhibitors will be removed by the extraction or isolation processes used.
- 2.2. Try additional re-extraction steps to further purify the samples, such as repeating or performing a phenol-chloroform extraction and/or ethanol precipitation.

- 2.3. Try sample clean-up using magnetic SPRI beads, columns or filters designed to purify/concentrate DNA.

Appendix – Primer Sequences for ordering

Notes:

- See Excel file [JGI iTag primer sequences v1.0.xlsx](#) for full length primer sequences for ordering.
- Sequences are in 5'→ 3' orientation and should be ordered in this orientation.
- Primers are used at a final 10µM concentration after resuspension
- Primers contain Illumina adapter sequences that are compatible with Illumina sequencing primers and index primers, so they do not require custom spike-in primers for sequencing.
- RV primers contain a 12bp barcode index sequence (i7 index) and staggered 0-3nt spacer sequences that provide sequence diversity at the start of sequencing read 1 when amplicons are pooled for sequencing.
- FW primers contain a random 5nt spacer sequence to provide sequence diversity at the start of sequencing read 2.
- iTag targeted amplification primer sequences are as follows (see excel file for full-length primer sequences):
 - 16S V4 region primers:
 - FW (515FB): GTGYCAGCMGCCGCGGTAA
 - RV (805RB): GGACTACNVGGGTWTCTAAT
 - 16S V4-V5 region primers:
 - FW (515F-Y): GTGYCAGCMGCCGCGGTAA
 - RV (926R): CCGYCAATTYMTTTRAGTTT
 - 18S V4 region primers:
 - FW: CCAGCASCYGCGGTAATTCC
 - RV: ACTTTCGTTCTTGATYRA
 - Fungal ITS2 region primers:
 - FW (ITS9): GAACGCAGCRAAIIGYGA
 - RV (ITS4): TCCTCCGCTTATTGATATGC

Protocol Change History

1. Version 1.0 – new protocol