

iTag Sample Amplification QC

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Summary

The purpose of this procedure is to QC samples prior to submitting them to the JGI for iTag 16S, 18S, and/or ITS sample prep and sequencing analysis. This sample QC is an amplification test to ensure that samples will be successful in JGI sample prep and that there are no contaminants present that will inhibit amplification.

Materials & Reagents

- Molecular Biology or PCR Grade Water, nuclease-free
- QuantaBio 5PRIME HotMasterMix (VWR Catalog# 10847-706 or 10847-708)
- Bovine Serum Albumin (BSA), Molecular Biology Grade
- PCR Primers for specific iTag application:
 - Sequences are in 5' →3' orientation and should be purchased in this orientation.
 - Primers are used at 10 μ M concentration.
 - 16S V4 region primers:
 - FW (515F): GTGCCAGCMGCCGCGGTAA
 - RV (805R): GGACTACHVGGGTWTCTAAT
 - 16S V4 region version 2 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): GAACGCAGCRAAIIIGYGA
- RV (ITS4): TCCTCCGCTTATTGATATGC
- 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 (or similar instrument).

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 10ng (10ng/μl) per 1X PCR reaction.

NOTE: All reagents/stock solutions should be defrosted 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. Prepare the template/primer mix in PCR tubes or 96-well plates depending on sample number and add the Molecular Biology Grade Water, BSA, and 5 PRIME HotMasterMix as specified in Table 1.

Table 1: Mix the following components at ambient temperature; volumes are for 1X PCR reaction.

Component	Volume (μl)
Molecular Biology Grade Water	12.0
BSA (10mg/ml)	1.0
5 PRIME HotMasterMix	10.0
Forward Primer (10μM)	0.5
Reverse Primer (10μM)	0.5
Template DNA (10ng/μl)	1.0
Total 1X reaction volume	25.0

- 1.2. Close the tube, or seal the plate, 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 a thermal cycler and start the PCR program using the cycling conditions in Table 2.

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

Table 2: PCR conditions for iTag sample amplification.

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

3. Assess Amplified Product for Quantity and Size

- 3.1. Run amplified product on agarose gel or Bioanalyzer (or similar instrument like LabChip or Fragment Analyzer) to ensure sample amplification occurred with the expected band size of (including primers): ~331bp for 16S V4, ~450bp for 16S V4-V5, ~431bp 18S V4, or 240-460bp for ITS2.
- 3.2. Samples showing proper amplification and sizing have passed the Amplification QC and are approved to ship to the JGI for iTag processing.
- 3.3. See **Troubleshooting** section for samples that fail to pass the Amplification QC.

Troubleshooting

Assuming no process issues occurred during the Amplification QC, 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 QC. 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.
- 1.3. If sample dilution works and allows for the sample to pass the Amplification QC, then please work with your JGI Project Manager to properly submit the diluted samples to the JGI for processing.
- 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. Please contact your JGI Project Manager for recommendations on extraction and isolation procedures.
 - 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.
 - 2.4. If further purification of the sample works and allows it to pass the Amplification QC, then please work with your JGI Project Manager to properly submit the purified samples to the JGI for processing.
- 3.**

Protocol Change History

1. Version 1.0 – new protocol; August 14, 2014
2. Version 1.1 – edit to Table 2 to split out the Step number to a separate column.
3. Version 1.2 – edit to add expected 18S V4 & ITS2 amplicon sizes in step 3.1; September 22, 2014.
4. Version 1.3 – added 16S V4-V5 primer info; April 18, 2016
5. Version 1.4 – Changed amplification kit to QuantaBio's 5PRIME HotMasterMix; September 1, 2016
6. Version 1.5 – Added 16S V4 version 2 primer info; September 8, 2017