**ROUGH PROTOCOL for ITS MiSeq SAMPLE PREP**

Created by C. Kenkel, last edited by Sarah W. Davies on May 19, 2015, updated by Dan Barshis on 3-Nov-16

This protocol was developed to amplify single sequence products via the MiSeq platform in order to gauge within-sequence diversity of the ITS2 region. However, this protocol can be applied to any amplicon sequencing application. As long as you have obtained high molecular weight DNA, this protocol should work. It is important to start with a large template amount in order to include all the sequence diversity that exists. Cycle checking avoids over-amplification of products, which can result in anomalous sequences and over/under-representation of specific products. PCR clean-up following the initial amplification removes “short” primers so that the barcoded MiSeq primers can be added without resulting in concatenated products in the second PCR step. Since the identity of each sample is preserved through the barcode sequence, samples can be pooled for sequencing.

1. Cycle check all samples using 30ul total volume PCR’s – you are looking for a cycle number that gives a distinct but FAINT band. Avoid overamplification! qPCR can also work but generally only gives a good cycle estimate when you have a single clade present (i.e. all are Clade C). If you think that your community contains >1 Clade qPCR does a poor job at quantifying the symbiont DNA present.
   1. You want a large amount of template (20-50 ng of target/reaction) in order to obtain sample diversity. This will lower your cycle number – start checking at 18-20 cycles.

**Master Mix Recipe for ExTaq non-pre-mixed**

Reagent 1x

H2O 12.6ul

10x ExTaq Buffer 2ul

10mM dNTPs 1.6ul #based on clontech’s datasheet

10uM F+R Primer Mix 2ul

ExTaq HS 0.10ul

~~PFU 0.15ul~~

Total: 18ul/rxn plus 2ul template

**Master Mix Recipe for pre-mixed Ex-Taq**

Reagent 1x

H2O 6ul

Pre-Mix ExTaq 10ul

10uM F+R Primer Mix 2ul

Total: 18ul/rxn plus 2ul template

**ITS2 PCR Profile**

95°C for 5min

15x 95°C for 40s

59°C for 2min

72°C for 1min

72°C for 10min

1. Add cycles as necessary to achieve a FAINT band for all samples. Test on agarose gel.
2. Clean amplified samples using Exo-SAP-it
3. For each cleaned sample, nanodrop and dilute in elution buffer as necessary to make concentration 10ng/ul for each sample.
4. Create new PCR to incorporate barcoded Illumina adapters

**Master Mix Recipe**

Reagent 1x

H2O 9.5ul

10x ExTaq Buffer 2ul

10mM dNTPs 0.5ul

ExTaq HS 0.1ul

PFU 0.1ul

Total: 12ul/rxn **plus**

3ul of 1uM barcoded Forward primer

3ul of 1uM barcoded Reverse primer

2ul of 10ng/ul template

**Barcode PCR Profile**

95°C for 5min

4x 95°C for 40s

59°C for 2min

72°C for 1min

72°C for 10min

1. Check all samples on gel (can add up to 1-2 more cycles to achieve band)
2. Once all samples run on same gel – “eyeball” product and decide how much to pool into final sample - we start with 5ul per sample and if one lane is 2x less bright, we add 10ul of it to the pooled sample tube, etc.
3. Clean amplified samples using PCR clean-up. Elute with 40 ul.
4. Run 20-40ul of eluted sample on gel. There should be a single band at your desired product size. We use SYBR green dye and illuminate on a blue light box.
5. Cut out band (target in this case is ~500 bp), soak in 10-20ul milli-Q (or other nuclease free) H2O overnight at +4C OR use a commercial gel extraction kit
6. Remove liquid => Sample is ready to be sequenced

**Example Primer Sequences**

It’s fairly simple to create your own barcoded primers. The target amplicon primers are slightly modified to include a linker that any of the remaining barcode primers can bind to. Barcodes can then be used for any amplicon target, so long as amplicon primers are properly modified. Dual barcoding is cost efficient –more samples with less barcodes – ex. 20 forward and 20 reverse barcodes can label up to 400 unique samples. See <https://wikis.utexas.edu/display/GSAF/Illumina+-+all+flavors> for more primer examples and barcodes.

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| --- | --- | --- |
| **Primer Name** | **Barcode** | Adaptor + Linker + **Pochon ITS2 Forward Primer** |
| ITS2-F-miseq | n/a | TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **GTGAATTGCAGAACTCCGTG** |
|  |  | Adaptor + Linker + **Pochon ITS2 Forward Primer** |
| ITS2-R-miseq | n/a | GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACAG **CCTCCGCTTACTTATATGCTT** |
|  |  | Illumina (Nextera) Primer + Barcode + Forward Adaptor |
| Hyb\_F13\_i5 | AGTCAA | AAT GAT ACG GCG ACC ACC GAG ATC TAC AC AGTCAA T CGT CGG CAG CGT C |
|  |  | Illumina (Nextera) Primer + Barcode + Reverse Adaptor |
| Hyb\_R43\_i7 | GCTGTA | CAA GCA GAA GAC GGC ATA CGA GAT GCTGTA GTC TCG TGG GCT CGG |