Protocol of ITS 2-steps PCR for Sequencing

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1. Dilute DNA samples

DNA samples need to be quantified and diluted to 2-3ng/μl with nuclease-free water. For DNA quantification, PicoGreen is preferred while NanoDrop may be used for high quality DNA (260/230 ≥ 1.7). The diluted DNA may be stored in PCR tubes or 96-well plates of your choice for easy use of multi-channel pipette.

2. 1stPCR

First, you need to test if this PCR condition works well for your samples using a small sample set (12) before proceed with many samples. This pre-testing also allows you to get familiar with the procedures.

To recover the diversity of microbial communities and limit potential artifacts from PCRs, all PCRs (both 1st and 2nd) are prepared in three replicates, each template DNA is 5ul,3ul, and 1 ul and each 25μl using recipes below. The 1st PCR uses regular primers, which is common for all samples.

Gene	Forward Primer (R1)		Reverse Primer (R2)	
	#	Sequence	#	Sequence
ITS	ITS7F	GTGARTCATCGARTCTTTG	ITS4R	TCCTCCGCTTATTGATATGC

1stPCR mix (25μl)

10× Buffer:	2.5 μl
Primer 515F 10 μM:	1 μl
Primer 806R 10 μM:	1 μl
Taq:	0.5 μl
Template DNA:	5 μl, 3ul and 1ul
Add water to a total volume :	25 μl

PCR program for 16s-1st PCR

	12 cycles for 1st				
94.0°C	94.0°C	52.0°C	68.0°C	68.0°C	4.0°C
1:00	00:20	00:25	00:45	10:00min	∞

3.Bead purification (see attached AgencourtAMPure XP product instruction, particularly pages 5-6 and highlighted parts)

After amplification, the PCR products from three replicates are combined and purified using 100μl bead solution to remove primer dimmers and other contaminants.

(1) Gently shake the AgencourtAMPure XP bottle (store at 4 ℃) to re-suspend any magnetic particles that may have settled.

(2) Add 100µl bead solutions to the combined PCR, gently pipette up and down 10 times to mix. (for large number samples, you may seal the wells well and vortex gently to mix)
(3) Incubate at room temperature without shaking for 5 minutes. Prepare fresh 70% ethanol (need 400 µl per sample).
(4) Place the samples on a magnetic plate for 2 min or until the supernatant has cleared.
(5) With the samples on the magnetic plate, carefully remove and discard the supernatant, do not disturb the ring of separated magnetic beads. Change tips between samples.
(6) With the samples on the magnetic plate, dispense 200 µl of 70% ethanol to each samples and incubate for 30 sec at room temperature. Aspirate out the ethanol and discard. Wash it once more. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of each well as ethanol is a known PCR inhibitor.
(7) With the samples still on the magnetic plate, allow the beads to air-dry for 10-15 min.
(8) Remove the samples from magnetic plate; add 50µl nuclease-free water to each sample. Gently pipette up and down 10 times to mix. (for large number samples, you may seal the wells well and vortex gently to mix)
(9) Incubate at room temperature for 2 minutes. Prepare 2nd PCR.
(10) Place the samples back to the magnetic plate for 2 minutes or until the supernatant has cleared.
(11) With the samples still on the magnetic plate, carefully transfer the 45ul (15µl is used as template for each replicate) products to 2nd PCR.

4. 2nd PCR mix (25µl)

Purified genes are recovered in 50µl water and 15µl is used as template for 25µl reaction in 2ndPCR. This time different barcode primers are used for each sample to distinguish sequences. The final PCR solutions combined and 4µl are examined on 1% agarose gel for presence and quality of target band.

2nd PCR mix (25 µl)

10× Buffer:	2.5 µl
Barcoded primers F/R 10/2 µM:	2 µl
Taq:	0.5 µl
Template DNA:	15 µl 1st PCR
Add water to a total volume:	25 µl

PCR program (Tm52.0°C for ITS)

	24 for 2nd PCR				
94.0°C	94.0°C	52.0°C	68.0°C	68.0°C	4.0°C
1:00	00:20	00:25	00:45	10:00min	∞

5. Picogreen:

Long-term storage (-20):

PicoGreen dye (100 µl each)

20X TE (pH 7.5, 200 mM Tris-HCl, 20 mM EDTA)

λ DNA standard (100 µg/mL) and some 50X diluted (2 µg/mL)

Short-term storage (4C)

One PicoGreen dye (to avoid freeze/thaw)

1X TE in DEPC treated water (50 ml-corning tube)

One λ DNA standard (2 µg/mL)

Sample preparation

a. 100X dilution on soil samples in TE or based on Nanodrop reading for dynamic range of 0-1000 ng/mL

1. Dye dilution: 200X dilution in TE right before the assay (only good for several hours after diluted)

2. Standards preparation (duplicates in 96-well plate): Sample standards preparation

Concentration(ng/ul)	λ DNA (2 µg/mL)	TE(ul)	diluted PicoGreen)(ul)
0	0	100	100
10	1	99	100
50	5	95	100
100	10	90	100
200	20	80	100
500	50	50	100

Standard curve: B, 0, 1, 5, 10, 20, 50, 100 µlλDNA (2 µg/ml)
PCR: 2 µl DNA to 98 µl 1× TE

3. Adding samples and standards first (200 µl TE for blanks), then mix with dye (in dark room)

4. Incubate for 2-5 minute in dark before reading in BMG Labtech FLUOstar OPTIMA

6. Quantify PCR products, pool samples, and gel purification

PCR products are quantified by PicoGreen, and equal amounts of DNA, typically 100 ng per sample, are combined to generate similar amounts of sequence number. The pooled library is loaded to 1% agarose gel, which should be run at least 1h at 96 volt to fully separate the target band from primer dimmers and contaminated bands. Sliced gel containing target genes is extracted using **QIAGEN Gel Extraction Kit** to removed agarose.
The procedures are as follow:

Gel purification (this is for 0.3g gel, read QIAGEN Gel Extraction Kit instruction)

(1) 1% gel slice 0.3g containing PCR products. Can be stored at -20°C for several days
(2) Add 0.9 ml QG buffer, 50°C invert to dissolve the gel completely. The color should be yellow
(3) Add 0.3ml is opropanol, invert to mix;

(4) To bind DNA, apply to column, vacuum. Add 0.5 ml QG to the column to further remove residual gel
(5) To wash, add 0.75ml buffer PE, wait for 2-5min, vacuum
(6) 13000rpm 1min to remove residual ethanol
(7) Column to clean 1.5ml tube, 50μl water to center, wait for 2-4min, 12000rpm 1min. PCR stored at -80°C