# 16S/ITS 2-step PCR

**1. Talk to Ying if you bring samples to the lab.**

**2. Dilute DNA samples**

DNA samples need to be quantified and diluted to 2 ng/µl using 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.

**3. 1st PCR, bead purification, and 2nd PCR**

First, you need to test if this PCR condition works for your samples using a small set of samples (12). Please show Ping your gel image before proceed with many samples. This pre-testing also allows you to get familiar with the procedures (e.g., how to use multichannel pipette, 96-well plates, beads purification).

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 25 µl using recipes below. The 1st PCR uses regular primers (e.g., 515F/806R for 16S), which is common for all samples. After amplification, the PCR products from three replicates are combined and purified using 75 µl bead solution to remove primer dimers and other contaminants (**see attached Agencourt AMPure XP product instruction, particularly pages 5-6 and highlighted parts**). Purified genes are recovered in 50 µl water and 15 µl is used as template for 25 µl reaction in 2nd PCR. This time different barcoded primers are used for each sample to distinguish sequences. The final PCR solution is combined and 4 µl are examined on 1% agarose gel for presence and quality of target band. **Please show Ping your gel images**.

| **1st PCR mix (25 µl)** | | **2nd PCR mix (25 µl)** | |
|---|---|---|---|
| 10× Buffer: | 2.5 µl | 10× Buffer: | 2.5 µl |
| Primer 515F 10 µM: | 1 µl | Barcoded primers F/R 10/2 µM: | 2 µl |
| Primer 806R 10 µM: | 1 µl | Homemade Taq: | 0.5 µl |
| Homemade Taq: | 0.5 µl | Template DNA: | 15 µl 1st PCR |
| Template DNA: | 5 µl (10-15 ng) | | |

Add water to a total volume of 25 µl

**PCR program (**For ITS, Tm 52.0°C and primers ITS7F and ITS4R)

| | **10 cycles for 1st and 20 for 2nd PCR** | | | | |
|---|---|---|---|---|---|
| 94.0°C | 94.0°C | 53.0°C | 68.0°C | 68.0°C | 4.0°C |
| 1:00 | 00:20 | 00:25 | 00:45 | 10:00min | ∞ |

**Bead purification (see attached Agencourt AMPure XP product instruction, particularly pages 5-6 and highlighted parts)**

**(1)** Gently shake the **Agencourt AMPure XP** bottle (store at 4 ℃) to resuspend any magnetic particles that may have settled.
**(2)** Add 75 μl bead solution to the combined PCR, gently pipette up and down 10 times to mix. (for large number samples, you may seal the wells well and voltex 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 50 μl 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 voltex gently to mix)
**(9)** Incubate at room temperature for 2 minutes. Prepare 2$^{nd}$ 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 products to 2$^{nd}$ PCR.

**4. 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. If some of your PCR products are less than 100 ng (e.g., 80 ng), bring the gel image and talk to Ping. The pooled library is loaded to 1% agarose gel. The gel needs to be run at least **1h at 96 volt** to fully separate the target band from primer dimers and other non-specific bands. Sliced gel containing target genes is extracted using **QIAGEN Gel Extraction Kit** to removed agrose. The procedures are as follow:

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

**Gel purification (this procedure is for 0.3g gel, you may scale up based on your gel weight, 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 isopropanol, 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

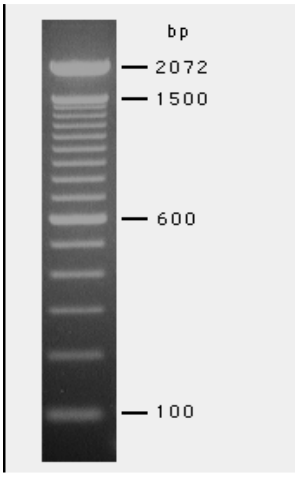
**5. Label your library clearly with readable handwriting and give it to Ping. Also, fill the attached sample information form and send it back to Ping before sequencing**

The following information is needed on the 1.5-ml centrifuge tube:
**On the tube cap**: (1) your first name
(2) gene (e.g., 16S, ITS, or other gene)
(3) sample number
**On the tube body**: (1) concentration (ng/µl) by Nonodrop
(2) date

**100 bp DNA Ladder** (100-1,500 bp, at 100 bp increments)

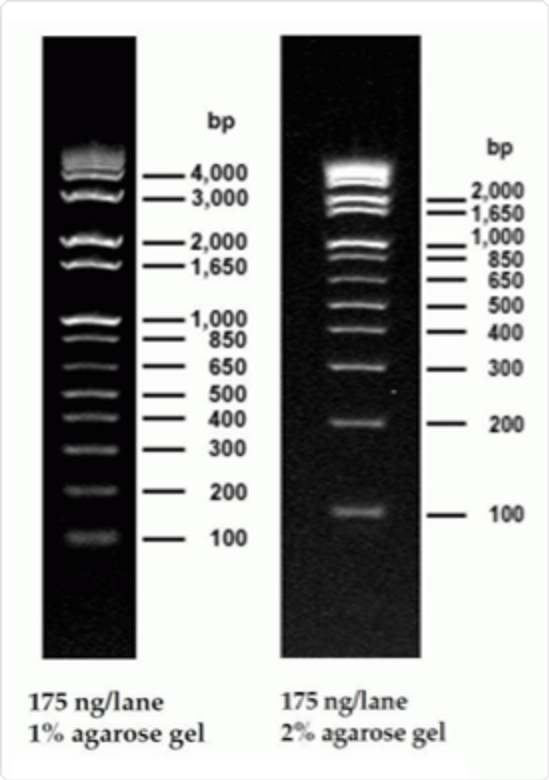
Invitrogen (Cat. # 15628-019, 50µg, 1µg/µl, -20°C). The 100 bp DNA Ladder is suitable for sizing double-stranded DNA from 100 to 1,500 bp (see picture below). The ladder consists of 15 blunt-ended fragments ranging in length from 100 to 1,500 bp, at 100-bp increments, and an additional fragment at 2,072 bp. The double-stranded ladder can be visualized on 1% to 2% agarose gels after ethidium bromide staining. For easy reference on agarose gels, **the 600- bp band is two- to three-times brighter than the other bands in the ladder**. Storage buffer: 10 mM Tris-HC1 (pH 7.5), 1 mM EDTA. Load approximately 0.1µg ladder/mm lane width.

**To prepare:** 50µl ladder (1 µg/µl), 150µl 6× dye (FC 25%), 400µl TE, total 600µl, load 5µl=0.42µg



**100 bp DNA Ladder, 0.5 µg/lane, 2% agarose gel stained with EthB**

Invitrogen 1KB plus DNA ladders




**Protist PCR:**

1) The protist suggested to be amplified using two steps PCR, detailed protocol just refer to that for 16S sequencing. the only difference from 16S sequencing is the annealing temperature and cycles due to the protist primer: 50 °C for 10 cycles at the first step, followed by 45 °C for 30 cycles at the second step.

3) Although 5 to 25ng DNA can be amplified in 25µL PCR system, 10 ng DNA was suggested.

2) We have two plates primers (96 x 2 = 192 barcode).