BIOC3301: Protocol for Metagenomics Analysis

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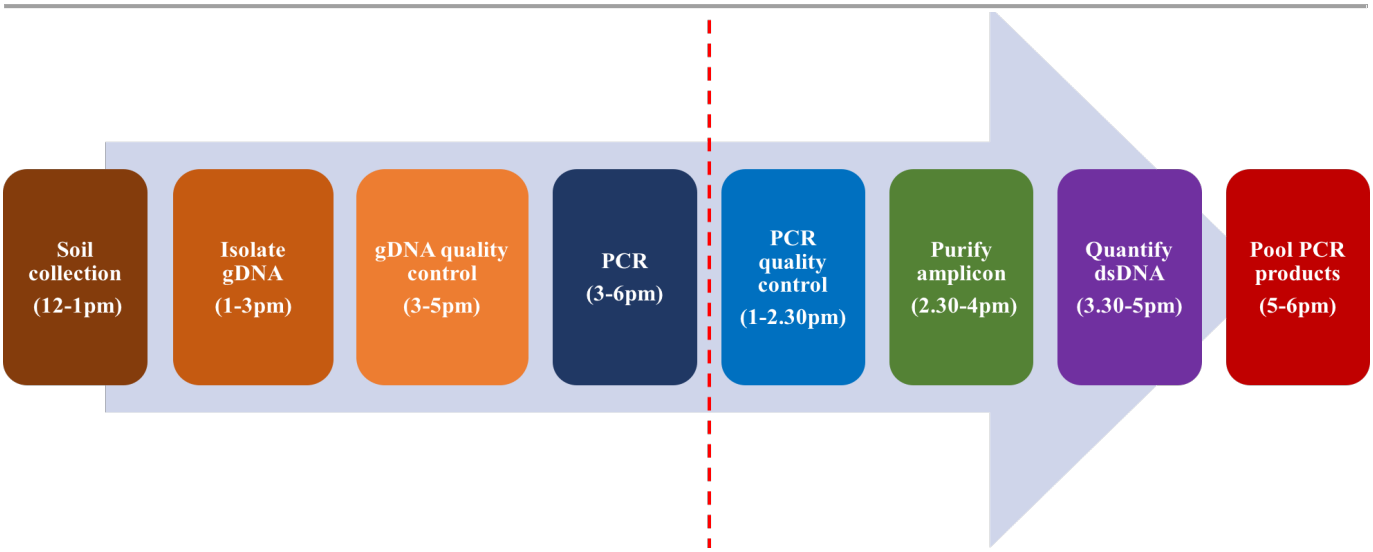

Figure 1: Overview of experiment. Line break at the point at which the PCR is running; this can be done overnight.

1: Collect soil from Gordon Square Garden

Precautions and factors to consider:

- Location: Gordon Square Garden, London, UK
- Ensure sampling GPS coordinates, date, time and conditions (temperature, weather) are noted
- Always wear gloves to avoid contaminating soil samples

Equipment and materials:

- Small shovel
- Bulb planter
- Gloves
- Falcon tubes (50 mL)

Procedure:

1. Generate map overview of Gordon Square and divide into 5 equal regions. **Note:** Systematic sampling is representative of entire park to avoid bias and increase chances of sampling within different conditions (e.g. different microbiomes, different chemical and physical soil conditions).
2. Generate axes for each region of the map.
3. Generate 2 random numbers (ensures unbiased samples) within the axes using a calculator and use as coordinates to locate the sampling site within the region of the map.
4. Use Global Positioning System to locate coordinate location. Location must be on soil and not on for example, a path. Otherwise repeat random generation of coordinates.
5. Use a bulb planter to take a soil sample at a depth of 10 cm. **Note:** Depth selected to avoid detritus present on or near the surface but to maintain a high bacterial presence, as this decreases with increased depth. Ensure to take samples at a consistent depth.
6. Transfer ~2 cm^3 of the sample to a 50 ml Falcon tube (keep each sample separated in a different container).
7. Label the sample with its region and number.
8. Note observable conditions for each sample site, including: sunlight/shading, moisture of soil, compactness of soil, vegetation, litter, animal remains, etc.
9. Use pH test strip to measure the pH of the soil sample.
10. Take a further 2 samples to act as controls.
11. Autoclave one control to act as the negative controls.
12. Add bacterial genomic DNA to the other control, to act as the positive control.
13. Store samples in a refrigerator.

2: Isolate genomic DNA from soil using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, CA, USA)

Precautions:

- Always wear gloves
- Solution C5 contains ethanol, which is flammable. Keep away from naked flames.

Equipment and materials:

- Microcentrifuge
- Pipettors and pipette tips
- Vortex and vortex adaptor
- PowerSoil® DNA Isolation Kit, which contains:
  - PowerSoil® Bead Tubes
  - PowerSoil® Solution C1
  - PowerSoil® Solution C2 - Inhibitor Removal Technology®
  - PowerSoil® Solution C3
  - PowerSoil® Solution C4
  - PowerSoil® Solution C5 - ethanol wash solution
  - PowerSoil® Solution C6 - 10 mM Tris
  - PowerSoil® Spin Filters - units in 2 mL tubes
  - PowerSoil® Collection tubes - 2 mL

Procedure:

1. Add 0.25 g of soil sample to **PowerSoil® Bead Tube**. If sample is of high water content, centrifuge at room temperature for 30 s at 10,000 g and remove water using pipette.
2. Gently vortex to mix the sample.
3. Check that **Solution C1** has not precipitated. If precipitated, heat solution to 60°C using a water bath until the precipitate has dissolved before use.
4. Pipette 60 µL of **Solution C1** to the sample and invert several times or vortex briefly.
5. Secure **PowerBead tubes** horizontally using the MoBio Vortex Adapter tube holder for the vortex. Vortex at maximum speed for 10 min. **Note:** If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes. If cells are difficult to lyse, a 10 min incubation at 70°C, after adding solution C1, can be performed.
6. Centrifuge **PowerTubes** at 10,000 g (~13,000 rpm) for 30 s at room temperature. **CAUTION**: Do not exceed 10,000 g or tubes may break.
7. Transfer supernatant to a clean 2 mL Collection Tube. Expect 400-500 µL of supernatant. Supernatant may have a dark appearance and still contain some soil particles).
8. Pipette 250 µl of **Solution C2** to the **PowerTubes** and vortex for 5 s.
9. Incubate tubes at 4°C for 5 min.
10. Centrifuge tubes at 10,000 g for 1 min at room temperature.
11. Avoiding the pellet, pipette up to 600 µL of supernatant to a clean 2 mL Collection Tube.
12. Add 200 µL of **Solution C3** to the tubes and vortex briefly.
13. Incubate at 4°C for 5 min.
14. Centrifuge the tubes at room temperature for 1 min at 10,000 g.
15. Avoiding the pellet, transfer up to (but no more than) 750 µL of supernatant into a clean 2 mL collection tube.
16. Shake to mix **Solution C4**.
17. Add 1.2 mL to the supernatant and vortex for 5 s. **CAUTION:** ensure solution does not exceed rim of tube.
18. Load ~675 µL of the collection tube solution onto a labelled **Spin Filter**.
19. Centrifuge at 10,000 g for 1 min at room temperature.
20. Discard the flow through and add an additional 675 µL of supernatant to the **Spin Filter**.
21. Centrifuge at 10,000 g for 1 min at room temperature.
22. Load the remaining supernatant onto the **Spin Filter**.
23. Centrifuge at 10,000 g for 1 min at room temperature.
24. Add 500 µL of **Solution C5**.
25. Centrifuge at room temperature for 30 s at 10,000 g.
26. Discard the flow through from the 2 mL Collection Tube.
27. Centrifuge at room temperature for 1 min at 10,000 g.
28. Carefully place **Spin Filter** in a clean 2 mL **Collection Tube**. **CAUTION:** Avoid splashing of **Solution C5**

onto the **Spin Filter**.

29. Add 100 µL of **Solution C6** to the centre of the white filter membrane.
30. Centrifuge at room temperature for 30 s.
31. Discard the **Spin Filter**. The gDNA is in the tube.
32. Store DNA at -20 to -80°C.

3: Check quality of gDNA by gel electrophoresis

Precautions:

- Always wear gloves

Equipment and materials:

- Pipettors and pipette tips
- Eppendorf tubes
- Agarose powder
- 12x14 cm horizontal gel electrophoresis device
- 1% ethidium bromide
- 5X loading dye

Procedure:

1. Measure 0.8 g of agarose powder.
2. Cast a ~100 mL **0.5% agarose gel** by mixing 0.8 g of agarose powder with 2 mL of 1X TAE buffer and 98 mL of distilled water in a microwavable flask.
3. Microwave flask in pulses with occasional swirling, for 1-3 min until the agarose is completely dissolved. **CAUTION:** do not over boil the solution.
4. Allow agarose solution to cool to ~50°C (hands can comfortably hold flask).
5. Add 0.5 µL of stock ethidium bromide to a final concentration of ~0.5 µg/mL.
6. Pour the agarose into a tray with the narrow well comb in its fixed position. Allow it to set at room temperature for 20-30 min (until the gel has completely solidified).
7. Once solidified, place the agarose gel into the electrophoresis unit/gel box.
8. Make 50 mL of 1X TAE buffer.
9. Carefully pour the buffer solution over the gel so that it covers the entire gel.
10. Transfer 1 µL of gDNA sample(s) (concentration between 50 ng – 500 ng) into clean, labelled tube(s). Bring the total volume up to 4 µL with 1X TE Buffer, pH 8.0.
11. Add 1 µL of 5X loading dye. The amount of loading dye should remain consistent among all samples to maintain even DNA migration on the gel.
12. Mix by pipetting up and down a few times.
13. Load onto gel according to the format listed below (where n is number of samples to be run):
    - ➢ Well 1: 10 µl of 1kB + ladder (Invitrogen), 100 ng/µL.
    - ➢ Well 2 to n: gDNA samples.
    - ➢ If DNA floats out of the well, some ethanol from Solution C5 may remain in the sample. Remove residual ethanol in a speed vacuum, desiccator, or air dry.
14. Run gel at **120V** until the dye line is ~70-75% of the way down the gel (~90 min).
15. Switch off power and disconnect the electrodes from the power source.
16. Carefully remove the gel from gel box.
17. Using a device that has UV light, take an image and visualise the DNA. **Note:** Expect the bands to appear streaked due to 'ungentle' bead treatment. Look for presence of smearing as this can indicate: (i) RNA contamination, (ii) DNA degradation, and (iii) impurities such as proteins and polysaccharides; these factors can inhibit chemical reactions during library construction.

4: Amplify the 16S rDNA V4 region by PCR using BioMix™ (BioLine Reagents Limited, London, UK)

Procedure:

1. Set up one large 72 µL master-mix and distribute into three tubes, according to table 1, as follows:
    A. Add 24 µL of the master-mix (sequences for primers in Appendix 1).
    B. Add 1 µL of isolated gDNA.

Table 1: Volumes For PCR Mixture		
Reagents	**Volume for 1X25 µL (µL)**	**Volume for 75 µL master-mix (µL)**
2X BioMix (contains reaction buffer, Mg^{2+}, dNTPs, *Taq* polymerase and additives)	12.5	37.5
515fB forward primer (5 pmol)	0.5	1.5
806rB reverse primer (5 pmol)	0.5	1.5
Template DNA	1.0	N/A
PCR grade water	10.5	31.5
Total	25.0	72.0

2. Add to thermocycler on set cycle. Thermal cycle programme:
    A. 94°C, 1 min
    B. 94°C, 15 s
    C. 50°C, 45 s
    D. 72°C, 30 s
    E. Repeat stages A-D 35 times
    F. 72°C, 5 min
    G. 4°C HOLD

IMPORTANT: Pool 3 samples (25 µL) into one tube prior to PCR quality control and purification.

5: Check quality of amplicons by gel electrophoresis

Precautions and factors to consider:

- Always wear gloves
- If gel has been prepared earlier, wrap in clingfilm and store in fridge

Equipment and materials:

- Pipettors and pipette tips
- Eppendorf tubes
- Agarose powder
- Tris-acetate-EDTA buffer
- Ethidium bromide
- 5X loading dye
- 12x14 cm horizontal gel electrophoresis device

Procedure:

1. Measure 1.5 g of agarose powder.
2. Cast a ~100 mL **1.5% agarose gel** by mixing 1.5 g of agarose powder with 2 mL of 50X TAE buffer and 98 mL of distilled water in a microwavable flask.
3. Microwave flask in pulses with occasional swirling, for 1-3 min until the agarose is completely dissolved. **CAUTION:** do not over boil the solution.
4. Allow agarose solution to cool to ~50°C (hands can comfortably hold flask).
5. Add 0.5 µL of 1% stock ethidium bromide to a final concentration of ~0.5 µg/mL.
6. Pour the agarose into a tray with the narrow well comb in its fixed position. Allow it to set at room temperature for 20-30 min (until the gel has completely solidified).
7. Once solidified, place the agarose gel into the electrophoresis unit/gel box.
8. Make 50 mL of 1X TAE buffer.
9. Carefully pour the buffer solution over the gel so that it covers the entire gel.
10. Transfer 1 µl of amplicon sample(s) into clean labelled tube(s) and bring the total volume up to 4 µL with 1X TE Buffer, pH 8.0.
11. Add 1 µL of 5X loading dye. The amount of loading dye should remain consistent among all samples to maintain even DNA migration on the gel.
12. Mix by pipetting up and down a few times.
13. Load onto gel according to the format listed below (where n is number of samples to be run):
    - ➢ Well 1: 10 µl of 1kB + ladder (Invitrogen), 100 ng/µL.
    - ➢ Well 2 to n: Amplicon samples.

14. Run gel for **30 minutes** at **120V** in 1X TAE buffer. **CAUTION:** Do not run for a long duration to prevent DNA running off the gel.
15. Switch off power and disconnect the electrodes from the power source.
16. Carefully remove the gel from gel box.
17. Using a device that has UV lights, take an image and visualise the DNA.
    Note: Analyse the gel photo for amplicon presence and its quality; the expected size is 384 bp. Using the standard molecular marker, make an educated estimate of the amount of product in the amplicon band, from this, establish a rough concentration of your amplicon.

6: Purify amplicon using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)

Precautions and factors to consider:

- Always wear gloves
- Label all tubes and filters
- SpinClean Buffer contains alcohol, so be careful not to wipe off the labels accidentally

Equipment and materials:

- PCR product tubes - 25 µL
- SpinBind - 125 µL per prep
- SpinClean Buffer - 10 mM Tris, 50 µL per prep
- Spin Filter unit
- 2 mL collection tube
- Microcentrifuge at 10,000 g
- Pipettors (volumes of 50 µL – 500 µL required)

Procedure:

1. Shake to mix the **SpinBind** before use.
2. Add 5X volumes of **SpinBind** to the PCR reaction mixture. For example, add 500 µL to 100 µL PCR reaction. Or, pool PCR products and then add the binding buffer.
3. Mix well by pipetting.
4. Label all the tubes and filters.
5. Transfer PCR/SpinBind mixture to a **Spin Filter unit**.
6. Centrifuge for 10-30 s at a minimum of 10,000 g (~13,000 rpm) in a table-top microcentrifuge.
7. Remove **Spin Filter basket** and discard the liquid flow-through from the tube by decanting.
8. Replace the Spin Filter basket in the same tube.
9. Add 300 µL **SpinClean Buffer** to the labelled Spin Filter.
10. Centrifuge for 10-30 s at a minimum of 10,000 g.
11. Remove **Spin Filter basket** and discard liquid flow-through by decanting.
12. Replace the Spin Filter basket back into the same tube.
13. Centrifuge for 10-30 s at a minimum of 10,000 g.
14. Transfer **Spin Filter** to a clean 2 mL collection tube.
15. Add 50 µL of **Elution Buffer** (10 mM Tris) solution provided or sterile water directly onto the centre of the white **Spin Filter** membrane.
16. Centrifuge 10-30 s at a minimum of 10,000 g.
17. Remove **Spin Filter basket** from the 2 mL collection tube.
18. Seal tube and store DNA at -20^{o}C.

7: Quantify dsDNA using SpectraMax® Quant™ AccuClear™ Nano dsDNA Assay – Explorer Kit (Molecular Devices, LLC, California, US)

Equipment and materials:

- Fluorescence microplate reader
- SoftMax Pro Software
- 96-well microplate (solid black)
- Vortex
- De-ionised water (buffer solution)
- dsDNA standard
- 100X AccuClear Nano dye

Workflow:

- ➢ Add sample and reagent
- ➢ Cover and mix
- ➢ Fluorescence measurement
- ➢ Data analysis

Procedure:

1. Warm reagents to room temperature before use.
2. Vortex reagents to minimise reagent loss in the cap, then centrifuge vials briefly.
3. Dilute 20X **AccuClear Nano Buffer** to 1X with dH_2O (3388 µL required, calculating for 3400 µL: 170 µL 20X buffer in 3230 µL dH_2O).
4. Dilute 100X dye (1:100) in 1X **AccuClear Nano Buffer** in a plastic container (**do not use glass**), and vortex to mix well (26 µL dye in 2600µL buffer).
5. Pipette 200 µL of working solution into each microplate well (total required is 2600 µL).
6. Prepare a set of dsDNA standards by diluting the 25 ng/µL dsDNA standard in the 1X AccuClear Nano Buffer as indicated in Table 2 below.
7. Pipette 10 µL of SpectraMax Quant AccuClear Nano dsDNA standards into separate microplate wells and mix.
8. Incubate microplate at room temperature for 5-10 min in the dark.
9. Measure fluorescence using a fluorescence microplate reader (SoftMax® Pro Software; excitation = 468 nm; emission = 507 nm).
10. Use standard curve (linear regression automatically computed by the plate-reader; a Molecular Devices SpectraMax i3) to calculate the concentrations of our DNA samples by using the RFU value from the reader. **Note:** If fluorescence of unknown sample exceeds the linear range, further dilute the sample and use 10 µL of diluted sample to perform the assay.

Table 2: Preparation of dsDNA Standards

Standard	Final Concentration	Standard Volume	1X AccuClear Nano Buffer Volume
A	25 ng/µL	100 µL of 25 ng/µL AccuClear Nano Standard	None
B	10 ng/µL	40 µL of 25 ng/µL AccuClear Nano Standard	60 µL
C	3 ng/µL	12 µL of 25 ng/µL AccuClear Nano Standard	88 µL
D	1 ng/µL	10 µL of 10 ng/µL	90 µL
E	1.3 ng/µL	10 µL of 3 ng/µL	90 µL

8: Pool PCR products for sequencing and final analysis

8 a) Pooling for a final DNA concentration of 10 nM

Use concentration results from dsDNA assay to adjust the **final DNA concentration to 10 nM** using the following calculation: (where: MW = number of bases [384] x average Mr of bases [660 $gmol^{-1}$ per base pair] = 253,440 $gmol^{-1}$; variables x, y, and z are unknown):

nM = ng/µL

$$10\ nM = \frac{ng/\mu L}{253{,}440} \times (1 \times 10^6)$$

$\therefore$ ng/µL = x ng/µL

Convert ng/µL to nM:

$$n = \frac{m}{Mr} = \frac{ng/\mu L}{253{,}440} = \left(\frac{ng/\mu L}{\frac{253{,}440\,gmol^{-1}}{10^{-6}\,\mu L}}\right) = y\ mol^{-1} = z\ nM$$

8 b) DNA sent for sequencing using Illumina MiSeq

The **Illumina MiSeq Reagent Kits v2 for 500 cycles (catalog ID: MS-102-2003)** was used.
This gives 250 cycles per read (each cycle is a nucleotide).

8 c) Analysis by computational methods

Use software, for example QIIME 1.9.1 (or current version).

Appendix

Appendix 1: Sequences Of Primers Used
Forward:
515fB PCR Primer Sequence: AATGATACGGCGACCACCGAGATCTACACGCT XXXXXXXXXXXX TATGGTAATT GT GTGYCAGCMGCCGCGGTAA (where: Y is C/T; M is A/C) Description: 5' Illumina adaptor, GoLay barcode, Forward primer pad, Forward primer linker, Forward primer
Reverse:
806rB PCR Primer Sequence: CAAGCAGAAGACGGCATACGAGAT AGTCAGTCAG CC GGACTACNVGGGTWTCTAAT (where: N is any; W is A/T) Description: Reverse Complement of 3' Illumina adaptor, Reverse primer pad, Reverse primer linker, Reverse primer (806rB)