Shoreline Complete™ V4 Protocol 1

Lyse, Purify, & Amplify Sets C1, C2, C3, C4, C5, C6 (16 Tubes)
Fecal and Tissue Samples

Rapidly purify microbial DNA from fecal and tissue samples and prepare library for sequencing using single step PCR amplification of the of the 254-base pair V4 region of the 16S rRNA gene for microbial community profiling. This sample kit is one of six that contain unique barcoded primer sets for processing 16 samples, each primer has the structure: ***<TruSeq Universal Adapter: Part A><8 base barcode><TruSeq Universal Adapter Part B><16S specific primer>***. Every sample will have a unique barcode at the completion of the PCR reaction for sequencing all the samples together.

Products

1. Shoreline Complete™ V4 Lyse, Purify, & Amplify Set C1…C6 (16 tubes) Cat. No. SCV4-C1…C6

Additional Items Needed for 16 Barcode Kits: (Not included; see Suggested Supplier list below)

1. Additional Items **Required** for Lyse and Purify
    a. 8-strip 0.2 mL PCR tubes w/individual domed caps for sample lysis and purification
    b. Sterile Laboratory Grade Water
    c. Fresh 70% ethanol
    d. TE Buffer - 10mM Tris, 1mM EDTA, pH 8.0
    e. 0.4M KOH Solution
    f. Magnetic Rack (‘bar’ or ‘post’ suggested for higher DNA yield)
2. Additional Items **Required** for Amplify:
    a. Qiagen MinElute® PCR Purification Kit
    b. Sterile Laboratory Grade Water
    c. Sterile 100% Ethanol
3. Suggested items for rapid processing
    a. Repeater Pipette with multi-volume tips
    b. Single Channel Manual Pipettes
    c. Multichannel Pipette 0.5-10 µL working volume
    d. Centrifuge with 96-well plate adaptor
    e. Sterile inoculating loops (16)
4. Suggested items for sample QC
    a. 0.8% agarose gel with ethidium bromide or other dsDNA stain
    b. Gel loading dye to mix with samples (e.g., NEB B7024)
    c. 2kb DNA ladder

Kit Contents for Shoreline Complete™ Lyse and Purify Protocol

1. Two 8-tube strips of dried Lysis-1
2. Purification Buffer (two 1.5 mL tubes with **green** cap)

Kit Contents for Shoreline Complete™ Amplify Protocol

1. 96-well PCR plate with 16 barcoded primers in wells A1-H2 (Columns 1 and 2)
2. 2X PCR Premix (**blue** cap)
3. PCR sealing film (1 sheet)

Important Considerations for Lyse and Purify:

1. Fecal samples should be thawed and stored on ice. Unmodified, frozen fecal samples will yield the best results. Storage temperatures can be -20°C to -80°C. If fecal sample has been diluted into buffer and stored at +4°C, significant degradation of the DNA in the sample can result in a few hours.
2. Shoreline Biome **lysis is pH dependent**. Samples stored in buffer with pH < 6.5 should use **at most** 10 µL input volume, otherwise the risk of suboptimal lysis affecting downstream results is high.
3. Fecal samples may contain pathogens. Handle samples using Universal Precautions. Please wear personal protective equipment and observe standard laboratory practices for handling, cleaning, and disposing of materials that come in contact with sample.
4. Shoreline Biome recommends including positive and negative controls in your project to monitor lysis and amplification efficiency as well as environmental contamination. Consider using mock microbiome cells (input=10^9) and mock microbiome DNA (input 10-100ng) as positive controls and water as negative control.
5. Shoreline Biome lysis results in ssDNA; for quantitation, please choose an appropriate kit (e.g., Qubit ssDNA assay kit #Q12012, Promega Quantifluor ssDNA system, #E3190)

Shoreline Complete™ Lyse and Purify Protocol

1. **Make 0.4M KOH**. Measure 0.135g KOH into 15 mL tube, then add 6 mL H_2O. 0.4M KOH expires after 7 days.
2. **Make 70% Ethanol**. Add 17.5 mL 100% ethanol to 7.5 mL sterile laboratory grade water
3. Remove **Purification Buffer** (Green Cap) from box and bring to room temperature.
4. **Sample Type:**
   - 4.1. **Frozen Solid Fecal Samples**
     - 4.1.1. Add **50 µL** Molecular Biology Grade water to each of the dried Lysis-1 tubes. Do not mix.
     - 4.1.2. Using sterile inoculating loop, transfer **~3 mg** (Fig. 1) of each fecal sample to the corresponding labeled tube. Spin each loop to disperse the sample in Lysis-1, and discard loop. Do not mix; **do not exceed 10mg of fecal sample**.
   - 4.2. **Mouse Fecal Pellet**
     - 4.2.1. Add **50 µL** Molecular Biology Grade water to each of the dried Lysis-1 tubes. Do not mix.
     - 4.2.2. Place up to 5 mg (1/4 mouse pellet) in each tube. **Do not add more than 10mg** (1/2 mouse pellet) Do not mix.
   - 4.3. **DNA Genotek OMNIgene®•GUT Sample Collection Tube**
     - 4.3.1. Add **10 µL** of sample from the OMNIgene®•GUT Sample Collection Tube to Lysis-1. Do not mix.
   - 4.4. **Liquid Fecal Samples stored in other buffers**
     - 4.4.1. Add **40 µL** Molecular Biology Grade water to each of the Lysis-1 tubes. Add **10 µL (maximum)** of sample. Do not mix.
   - 4.5. **Tissue Samples**
     - 4.5.1. Add **50 µL** Molecular Biology Grade water to each of the dried Lysis-1 tubes. Do not mix.
     - 4.5.2. Place up to 20 mg (up to 30mg) tissue sample in Lysis-1 tubes. Do not mix.

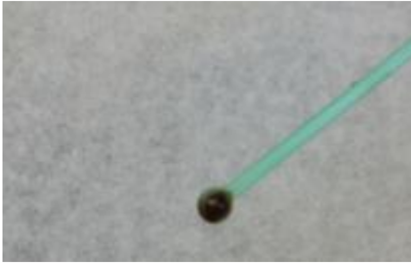

Figure 1 (left) Image shows 2 mg fecal material on a 1 µL inoculating loop.

Figure 2 (right) Precipitated material after being spun down from lysis step

5. **Add 50 µL 0.4M KOH** to each tube of Lysis-1/sample mix. Do not mix. (A precipitate will form.)
6. Cap samples tightly; set PCR machine for proper tube height, close and lock the lid, and **heat samples to 95°C for 5 minutes**. Precipitate will dissolve. After incubation, **allow sample to cool to RT before unlocking PCR lid** – failure to do so may result in lids popping off tubes. **While samples are cooling**:
   6.1. Label **clean 200 µL PCR tubes** for each sample
   6.2. Vortex Purification Buffer tube (Green Cap) to re-suspend any brown magnetic beads that may have settled
   6.3. Add **50 µL of Purification Buffer** (Green Cap) to each ***clean*** tube for sample purification
7. **Transfer Lysis-1 samples from PCR machine to ice for 2 minutes**. Precipitate will re-form.
8. **Remove Lysis-1 samples from ice, and spin for 60 seconds** in mini-centrifuge to pellet precipitate (Fig. 2)
9. Carefully **transfer 50 µL** of supernatant **into the clean tubes** containing 50 µL of Purification Buffer prepared as per the instructions in step 7.2 above; and **pipette up and down to mix**.
10. Cap Lysis-1/Purification Buffer samples tightly; set PCR machine for proper tube height, close and lock the lid, and **heat samples to 50°C for 3 minutes** to allow DNA to bind to beads.
11. **After 50˚C incubation, place sample tubes on magnetic rack** to pellet beads with bound DNA to side of the tube (~60 seconds).
12. Once all beads are pelleted, **remove all supernatant SLOWLY and discard.** Avoid aspirating beads.
13. **Remove tubes from magnet**, **add 100 µL of 70% ethanol** (at room temperature) to each tube, and re-suspend beads by pipetting gently up and down.
14. **Return tubes to magnet and allow beads to pellet** (~30 seconds)
15. **Pipette off residual ethanol and let air dry for 2 - 3 minutes**. Over-drying will make eluting DNA more difficult, so watch to ensure ethanol is mostly gone but bead pellet does not begin to shrink and crack.
16. **Remove tubes from magnet**
17. Add **20 µL of 1X TE buffer** to tubes and briefly pipette up and down to re-suspend beads. Solution should turn brown in color to reflect beads mixed homogeneously throughout.
18. **Let solution incubate at room temperature for 2 minutes**
19. **Place tubes on magnetic rack** for 30 - 60 seconds to pellet magnetic beads. **DNA is now in solution.**
20. Transfer supernatant containing eluted DNA into **clean** labeled microcentrifuge tubes on ice, avoiding pelleted beads.
21. **Remove tubes with pelleted beads from rack.**
22. **Add another 20 µL 1X TE buffer** onto bead pellet, gently pipette up and down to suspend beads.
23. **Incubate tubes for 2 minutes at room temperature.**
24. Return tubes to magnetic rack. Allow beads to pellet to sides for 30 - 60 seconds.
25. **Remove supernatant containing DNA** and combine with first eluate in the labeled tubes, avoiding pelleted beads.

26. **Dilution:**
    - 26.1. **For tissue Samples: Do not dilute. Store DNA at +4°C**. This is your **Shoreline Biome Lysis Mix**.
    - 26.2. **For solid fecal, liquid fecal, mouse pellet or DNA Genotek OMNIgene®•GUT Sample Collection Tube fecal samples: Dilute** samples 1:5 by **adding 160 µl 1X TE buffer** to 40 µl of combined eluted DNA for a total of 200 µl. **Store DNA at +4°C**. This is your **Shoreline Biome Lysis Mix**.

NOTES: DNA is **denatured**, due to KOH treatment during lysis. Although Lysis Mix may retain contaminants, the Shoreline Complete™ Amplify protocol is designed to work in the presence of these contaminants. Store Lysis Mix at +4°C for up to 10 days, or frozen at -20°C for 3 months. Avoid repeated freeze/thaws.

Shoreline Complete™ Amplify Protocol

NOTE: Only Columns 1 and 2 will be used.

1. Remove the plate seal from the Amplicon Primer plate.
2. **Add 10 µL** 2X PCR Premix (**blue** cap) to Columns 1 and 2 of the 96-well plate.
3. **Add 10 µL Shoreline Biome Lysis Mix** to each of the Column 1 and 2 wells.
4. Using the new PCR sealing film provided with the kit, cover the plate and seal tightly with no gaps.
5. Primers will dissolve off the bottom of each well. Observe that blue color is uniformly distributed throughout 20 µL reaction, if not, tap plate gently until uniform blue color is achieved in each well.
6. Spin plate briefly if reaction mix is not completely at the bottom of the wells.
7. Transfer the plate to PCR machine; close and lock the lid. Run PCR protocol:

95°C, 3:00	Denaturation
Repeat 34X	95°C, 0:20 58°C for 0:15 **(Ramp speed 4°C/sec)** 72°C, 0:15
72°C, 2:00	Final extension

8. Check reactions on gel: Run 1.5 µL of sample with 5 µL diluted gel loading dye on 0.8% agarose gel in TBE, 150V for approximately 45 minutes, with DNA ladder. Band should be present at ~ 450bp.

Qiagen MinElute® PCR Purification Protocol

1. Follow MinElute instructions for adding Ethanol (96% - 100%) to PE buffer, and label tubes.
2. Pool 5 µL of each amplified sample from the 96-well plate into a clean 1.5 mL microcentrifuge tube. **(Save 2 µL of pooled unpurified DNA for gel comparison with purified DNA in the last step below)**
3. Combine **78 µL** of pooled sample with 400 µL of Qiagen PB buffer.
4. Transfer **all** the resulting solution into the MinElute column with provided 2 mL collection tube.
5. Centrifuge the MinElute column at 17,900 RCF for 1 minute or until solution has passed through the column. Discard the flow-through in the collection tube and return the column into the empty tube.
6. Add 750 µL Buffer PE to the MinElute column and centrifuge at 17,900 RCF for 1 minute; discard flow through and return MinElute column to the collection tube. Centrifuge for 1 minute at 17,900 RCF to remove residual Ethanol.
7. Place MinElute column in a clean 1.5 mL microcentrifuge tube.
8. Add 50 µL EB Buffer (10mM Tris-Cl, pH 8.5) or water directly onto center of the MinElute membrane. Incubate column for 1 minute at RT, and then centrifuge column at 17,900 RCF for 1 minute.

9. Run 2 µL of purified DNA with 5 µL of diluted gel loading dye next to 2 µL pooled DNA^{++} with 5 µL gel loading dye on 0.8% agarose gel for 60 minutes at 100V to verify the purity of the DNA sample with no primer carry-over or contaminating gDNA.
10. This is your purified amplicon pool ready for Illumina sequencing.

Suggested Suppliers

Qiagen: MinElute® PCR Purification Kit (Cat. No. 28004)

BD Worldwide: (Sterile) Calibrated Disposable Inoculating Loops, Green, #220214

Fisher Scientific: Biotech Grade Water BP24854; Potassium Hydroxide P250; TE BP24731

Thermo Fisher: Invitrogen Dyna Mag 96 side magnetic rack, #12331D

Permagen: 0.2 mL PCR Strip Magnetic Separation Rack 8 or 12 Strip, Cat #MSR812

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