Shoreline Complete™ V4 Protocol 1

Lyse, Purify, & Amplify Sets A, B, C (96 wells)
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 up to 288 samples. This kit is one of three that contain unique barcoded primer sets for processing 96 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

Up to 288 different DNA samples can be barcoded and combined in a single sequencing run using the three available kits.

1. Shoreline Complete™ V4 Lyse, Purify, & Amplify Set A (96 Wells) Cat. No. **SCV4-A**
2. Shoreline Complete™ V4 Lyse, Purify, & Amplify Set B (96 Wells) Cat. No. **SCV4-B**
3. Shoreline Complete™ V4 Lyse, Purify, & Amplify Set C (96 Wells) Cat. No. **SCV4-C**

Additional Items Needed for 96 Barcode Kits: (Not included; see Suggested Supplier list below)

1. Additional Items **Required** for Lyse and Purify
   a. Two (2) sterile 96-well PCR plates
   b. Plate sealing film
   c. Sterile Laboratory Grade Water
   d. Fresh 70% ethanol
   e. TE Buffer - 10mM Tris, 1mM EDTA, pH 8.0
   f. 0.4M KOH Solution
   g. 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 (96)
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. Lysis-1 (96-well PCR plate, dried solution in each well)
2. Purification Buffer (two 8 mL bottles with **white** caps)

Kit Contents for Shoreline Complete™ Amplify Protocol

1. 96-well PCR plate with 96 barcoded primers in wells A1-H12
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. Bring Purification Buffer (**white** capped bottle) to room temperature.
4. Remove film covering the 96-well PCR plate containing dried Lysis-1.
5. **Sample Type:**
   - 5.1. **Frozen Solid Fecal Samples**
     - 5.1.1. Add **50 µL** Molecular Biology Grade water to each well in the 96-well Lysis-1 plate. Use of a repeater or multichannel pipette is recommended to save time. Do not mix.
     - 5.1.2. Using sterile inoculating loop, transfer **~3 mg** of each fecal sample to the corresponding labeled tube. (Fig. 1 and Fig. 2). Spin each loop to disperse the sample in Lysis-1, and discard **loop.** Do not mix; **do not exceed 10mg of fecal sample**.
   - 5.2. **Mouse Fecal Pellet**
     - 5.2.1. Add **50 µL** Molecular Biology Grade water to each well in the 96-well Lysis-1 plate. Use of a repeater or multichannel pipette is recommended to save time. Do not mix.
     - 5.2.2. Place up to 5 mg (1/4 mouse pellet) in each well. **Do not add more than 10mg** (1/2 mouse pellet) Do not mix.
   - 5.3. **DNA Genotek OMNIgene®•GUT Sample Collection Tube**
     - 5.3.1. Add **10 µL** of sample from the OMNIgene®•GUT Sample Collection Tube to Lysis-1. Do not mix.
   - 5.4. **Liquid Fecal Samples stored in other buffers**
     - 5.4.1. Add **40 µL** Molecular Biology Grade water to each well in the 96-well Lysis-1 plate. Add **10 µL (maximum)** of sample. Do not mix.
   - 5.5. **Tissue Samples**
     - 5.5.1. Add **50 µL** Molecular Biology Grade water to each well in the 96-well Lysis-1 plate. Use of a repeater or multichannel pipette is recommended to save time. Do not mix.

5.5.2. Place up to 20 mg (up to 30mg) tissue sample in each well. Do not mix.

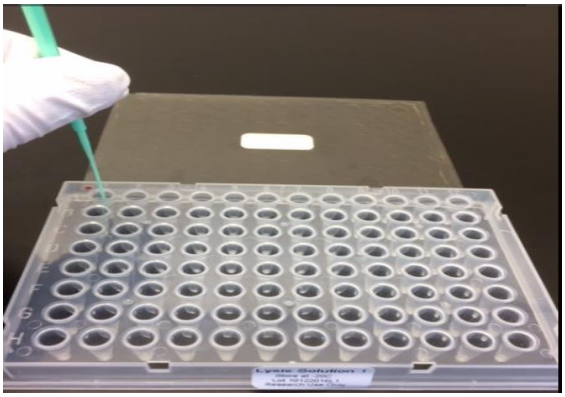

Figure 1.

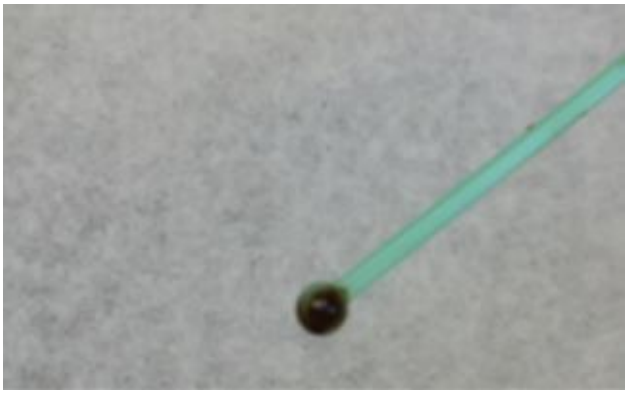

Figure 2.

6. **Add 50 µL 0.4M KOH** to each well position in the 96-well plate. Use of a repeater or multichannel pipette is recommended to save time. A precipitate will form in the wells. Do not mix.
7. **Cover the sample plate with PCR film,** load the sample plate into the PCR machine, close and lock the lid, and **heat samples to 95°C for 5 minutes**. Precipitate will dissolve.
8. **Place sample plate on ice for 2 minutes.** Precipitate will re-form.
9. **While samples are on ice**:
   9.1. Vortex Purification Buffer bottle (**white** cap) to re-suspend any brown magnetic beads that may have settled
   9.2. **Add 50 µL** of **Purification Buffer** to each well of a ***clean*** 96-well plate for sample purification.
10. **After incubating sample plate on ice, spin sample plate** in plate centrifuge at 2000 RCF for 3 minutes (or 400 RCF for 7 minutes) to pellet precipitate to bottom of wells (see Figure 3 below).

Figure 3. *Pelleted precipitate in bottom of wells after spin*

11. Carefully **transfer 50 µL** of supernatant **into the appropriate well** of the clean 96-well plate with Purification Buffer prepared as per the instructions in step 9 above; and **pipette to mix**.
12. **Cover the sample plate with a *new* PCR film**, load sample plate onto the PCR machine, close and lock the lid, and **incubate solution for 3 minutes at 50˚C** to allow DNA to bind to beads
13. After incubation, place plate on magnetic rack to pellet beads with bound DNA (~60 seconds), carefully remove PCR film.
14. Once all beads are pelleted, **remove all supernatant SLOWLY and discard.** Avoid aspirating beads.
15. **Remove plate from magnet**, **add 100 µL 70% ethanol** to each well, and re-suspend beads by pipetting gently up and down.
16. **Return plate to magnet and allow beads to pellet** (~30 seconds)

17. **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.
18. **Remove plate from magnet**
19. Add **20 µL of 1X TE buffer** to each well and briefly pipette up and down to re-suspend beads. Solution should turn brown in color to reflect beads mixed homogeneously throughout.
20. **Incubate plate at room temperature for 2 minutes**
21. Place plate on magnetic rack for 30 - 60 seconds to **pellet magnetic beads**. **DNA is now in solution.**
22. **Transfer supernatant containing eluted DNA** into a **clean** PCR plate on ice, avoiding pelleted beads.
23. **Remove plate with pelleted beads from rack.**
24. **Add another 20 µL 1X TE buffer** onto bead pellet, gently pipette up and down to suspend beads.
25. **Incubate plate for 2 minutes at room temperature.**
26. Return plate to magnetic rack. Allow beads to pellet to sides for 30 - 60 seconds.
27. **Remove supernatant containing DNA** and combine with first eluate in clean plate, avoiding pelleted beads.
28. **Dilution:**
    - 28.1. **For tissue Samples: Do not dilute. Store DNA at +4°C**. This is your **Shoreline Biome Lysis Mix**.
    - 28.2. **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

1. Remove the plate seal from the Amplicon Primer plate.
2. **Add 10 µL** 2X PCR Premix (**blue** cap) to each position in the 96-well plate. (Use a Repeater pipette.)
3. **Add 10 µL Shoreline Biome Lysis Mix** to each of the 96-wells.
4. Using the 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 *(when pooling multiple plates, pool 5 µL of each amplified sample from all wells of all plates)* 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 **80 µL** of pooled sample with 400 µL of Qiagen PB buffer.
4. Transfer **all** of 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 unpurified 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

Dot Scientific Inc.: 96-well plate, #353-PCR or #354-PCR

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

Thermo Fisher: Invitrogen Dyna Mag 96 side magnetic rack, #12331D or Magnetic stand-96 #AM10027