Equipment and supplies needed:

- Paasche H-100D single action airbrush & compressor package (Amazon.com)
- 1.5 ml conical microcentrifuge tubes (Fisher Scientific 05-408-129)
- Sterivex GP 0.22-µm filter units (Millipore SVGP01015)
- Disposable 10 ml needle-less syringes with Luer-Lok tips (BD Medical 309695)
- Aluminum weigh dishes (Fisher Scientific 08-732-104)
- Percoll (GE Healthcare 17-0891-01)
- DNeasy Blood and Tissue DNA extraction kit (Qiagen 69504)
- Genomiphi V2 DNA amplification kit (GE Healthcare 45001222)
- Floor model centrifuge & rotor for 50 cc tubes (Avanti JE Centrifuge, JLA 10.500 rotor, Beckman Coulter)
- Refrigerated benchtop microcentrifuge (Eppendorf Centrifuge 5804R, Fixed angle rotor FA-45-30-11 with aerosol-tight lid)

Common laboratory materials (beakers, forceps, razor blades, squirt bottles, 50 cc tubes, 15 cc tubes etc.)

Any use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Preparation (before beginning protocol):

Solutions (sterilize by autoclaving):

10X PBS (500 ml)	**PBSE (500 ml)**	**Qiagen Lysis Buffer (300 ml)**
40 g NaCl 1 g KCl 7.2 g Na_2HPO_4 1.2 g KH_2PO_4 in 400 ml of DI water adjust to pH 7.4 with HCl	50 ml 10X PBS 10 ml EDTA (0.5 M, pH 8.0) 440 ml DI water	12 ml Tris-Cl (0.5 M, pH 8.0) 1.2 ml EDTA (0.5 M, pH 8.0) 3.6 ml Triton X 100 283.2 ml DI water (Note: 20 mg/ml lysozyme will be added to an aliquot of this sterilized buffer immediately before use)

Autoclave to sterilize (wrap in foil):

- Several 500 ml glass beakers
- Several pairs of forceps
- Small hammer
- Large container of 1.5 ml tubes (need minimum of 76 per sample)
- Aluminum weigh dishes
- Ceramic mortar & pestle
- Glass reservoir for airbrush (before autoclaving, clean with bleach followed by sodium thiosulfate rinse to remove bleach; or wash well with soap and water followed by ethanol rinse)

Lid for glass reservoir is not autoclavable and must be sterilized by wiping with 70% ethanol

Set up:

- Turn on both centrifuges, set to 4˚C so they will cool down
- Turn on water baths, hot blocks, or incubators; set one to 37˚C and set another to 56˚C
- Remove coral samples in RNAlater from freezer and let them thaw at room temperature, if needed (not all freeze)
- For each sample, set up a rack of thirty sterile 1.5 ml tubes, filling each tube with 1 ml of 100% Percoll (using a 10 ml pipet). Keep tubes refrigerated until step 4.

Protocol:

It is possible to do one or two samples at a time. If the samples are whole coral branches, you will need to use flame-sterilized forceps to remove a branch from the tube of RNAlater and place it into the sterile mortar. Use the sterile pestle to fracture the coral and break open the calyces to expose the polyp tissue. You want about 1.5 – 2 polyps worth of tissue for this extraction. If the coral was fragmented before being placed into RNAlater, remove approximately two polyps worth of fragments from the slurry, place them into a sterile 500 ml glass beaker and proceed.

1) *Use airbrush to remove coral tissue from skeleton*
    a. Fill the sterilized airbrush reservoir with sterile PBSE buffer. Use sterile forceps to hold coral fragments inside the beaker and use airbrush to remove coral tissue and mucus from skeleton. Once the tissue is removed, pieces of skeleton can be discarded. This will generate a slurry of tissue, mucus, and PBSE in the beaker.
    b. Use a 10 ml needle-less syringe to aspirate resulting tissue slurry. Expel and aspirate the slurry between the syringe and beaker several times, until large tissue chunks have been broken apart.
    c. Use syringe to transfer slurry from beaker into a sterile 50 ml tube.
    d. Add PBSE buffer to bring the total volume of sample up to 35 ml.
    e. Tighten lid and shake tube vigorously for 5 min to disrupt the tissue.

2) *Low speed centrifugation (*Avanti JE Centrifuge, JLA 10.500 rotor, Beckman Coulter)

This step is intended to remove the sheared coral tissue fragments. You want to keep the supernatant, which contains the microorganisms, and avoid the pellet (coral bits).

    a. Place 50 ml tube containing 30 ml sample into rotor. Centrifuge at 4˚C, 2000xg, for 15 minutes.
    b. Check to make sure large tissue chunks have pelleted. If any pieces are still floating, repeat centrifugation.

3) *High speed centrifugation (*Eppendorf Centrifuge 5804R, Fixed angle rotor FA-45-30-11)

This step is intended to pellet all the microorganisms in the sample. This time you want to keep the pellet, which contains the microorganisms, and discard the supernatant.

a. Being careful not to disturb the pellet, use a P-1000 pipet to split the 30 ml supernatant from step 2 into thirty 1.5 ml tubes (One ml of supernatant per tube). If you are doing two samples, keep the second 50 ml tube at 4˚C in the refrigerator while performing this step on the first sample. You can split the second sample into tubes while the first sample is in the centrifuge.
b. Centrifuge the 30 tubes at high speed (≥ 20,000xg), 4˚C, for 20 minutes.
c. Pour off supernatant. (There will be a small amount of liquid that stays in the conical bottom of each tube. That is ok).
d. Resuspend pellets containing microbe fraction in 250 µl PBSE per tube.

4) *Percoll fractionation to remove remaining coral cells*
[Modified from Schleper et al., 1998, Journal of Bacteriology 180(19): 5003-5009].

a. Get the rack of 30 centrifuge tubes containing 1 ml each of 100% Percoll out of the refrigerator. Pair each Percoll tube to a sample tube (30 of each).
b. Using a P-1000 pipet, gently layer the ~250 µl of resuspended pellet onto the top of each tube of Percoll. Do this by adding the sample one drop at a time, running the drop down the inside of the tube containing the Percoll, so the sample rests on top of the denser liquid.
c. Centrifuge at 7000xg, 4˚C, for 20 minutes.
d. Use P-1000 pipet to remove the Top 250 µl from each of the 30 tubes into a 15 ml tube labeled "TOP" and then the next 250 µl into a second tube labeled "MID". Combine all 30 Tops together (in one tube) and Mids together (in a second tube), resulting in ~7 ml total volume per sample.
    i. Check sample via epifluorescent microscopy to determine which fraction has the cleanest and highest amount of microbes. Typically it will be the Top fraction, in which case the Mid fraction can be discarded.

5) *Cell Capture*

a. Filter the ~7 ml of the Top extraction through a Sterivex 0.22-µm filter to concentrate microbes on the filter. This is done by attaching a sterile 10 ml syringe to the top of the Sterivex filter, pouring the 7 ml sample into the syringe and then inserting the plunger to force the liquid through the filter and into the 15 ml tube below.
b. Detach the syringe from the filter, remove the plunger, refill with the filtrate, insert plunger, filter. Repeat one more time (for a total of three passes of the liquid through the Sterivex filter).
c. The liquid can now be discarded. The microbes are on the filter.

6) *DNA Extraction*

The removal of the filter from the Sterivex cartridge is best done in a sterile environment like a laminar flow hood. After the filter is removed, the extraction is done using the Qiagen DNeasy extraction kit, following the Gram-positive bacteria protocol, but the reagent volumes are doubled in the initial steps [i.e., steps **h** to **l** below are steps 2 to 6 in the Qiagen Gram-positive protocol with volumes doubled; steps **m - p** are steps 4 to 7 of the Qiagen Animal Tissue protocol, using normal volumes]. Before beginning this step, make lysis buffer: 1- 2 samples, you will need to add 0.06g of powdered lysozyme to 3.24 ml of lysis buffer. Mix gently to dissolve or it will foam.

a. Remove Sterivex filter from 10 ml syringe. Place filter cartridge in sterile aluminum weigh dish.
b. Holding the input port of the Sterivex filter cartridge (the end the syringe was previously connected to), use flame-sterilized hammer to hit the seam at the opposite end of the cartridge to break the plastic outer case loose from the filter.
c. Use flame-sterilized forceps to pull the inner filter cartridge out and place it in a clean, sterile aluminum weigh dish.
d. Use clean, ethanol-sterilized razor blade to cut the filter on both sides of the visible seam.
e. Use flame-sterilized forceps to peel the filter off the cartridge (this may come off in one large piece or multiple smaller fragments).
f. Use ethanol-sterilized razor blade to cut filter into quarters (four roughly equal amounts).
g. Use flame-sterilized forceps to place each of the four filter fragments into a separate sterile 1.5 ml tube (total of four tubes per sample).
h. Add 360 µl of lysis buffer (after adding lysozyme) to each of the four tubes.
i. Incubate tubes at 37˚C for 30 min. Vortex tubes every 10 min for 5 sec during this incubation to make sure filters are saturated.
j. Add 50 µl Proteinase K and 400 µl Buffer AL (without ethanol) to each of the four tubes. Mix by vortexing.
k. Incubate tubes at 56˚C for 30 min. No vortexing.
l. Remove liquid from each of the four tubes to four new tubes. Pulse the old tubes in a microcentrifuge for 30 sec to release any remaining liquid. Transfer that liquid to new tubes also. Discard old tubes containing filter fragments. Add 400 µl of 99-100% ethanol to each tube and vortex. (Ethanol addition can cause a precipitate to form and if filters remained in the tubes, it could stick to them and be lost).
m. Pipet the mixture from each tube in the previous step to a DNeasy Mini spin column (one column per sample, so all four tubes will be combined onto one spin column). The spin column can hold ~ 750 µl per centrifugation, so it will take seven rounds of centrifugation to get all of the liquid onto the column. Centrifuge at 10,000xg for 1 min. Discard flow through liquid. Repeat until all liquid from the four tubes has been put through the column. Discard collection tube after final spin.

n. Place DNeasy Mini spin column in a new 2 ml collection tube (provided in kit), add 500 µl Buffer AW1 and centrifuge for 1 min at 10,000xg. Discard flow through and collection tube.
o. Place DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2 and centrifuge for 3 min at 20,000xg (14,000 rpm) to dry the DNeasy membrane. Discard flow through and collection tube.
p. Place the DNeasy Mini spin column in a clean 1.5 ml tube and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 10,000xg to elute. [The DNA can be frozen at this point until ready to proceed.]

7) *Metagenome amplification*
   a. Using the Genomiphi V2 kit, follow manufacturer's instructions. Use the denaturation step.
   b. Do in triplicate, combine. Run 1 µl on a gel to check for amplification.
   c. Combine and purify the triplicates using the Qiagen PCR Purification kit, eluting in 50 µl.

8) *Check DNA quality*
   a. Run a 16S PCR using primers 63F/1542R to check for quality and presence of bacterial DNA versus coral DNA [See Galkiewicz & Kellogg, 2008, Applied and Environmental Microbiology 74: 7828-7831].