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Comparison of Three DNA Extraction Kits to Establish Maximum Yield and Quality of Coral-Associated Microbial DNA

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Open-File Report 2014-1066

U.S. Department of the Interior
U.S. Geological Survey

U.S. Department of the Interior
SALLY JEWELL, Secretary

U.S. Geological Survey
Suzette M. Kimball, Acting Director

U.S. Geological Survey, Reston, Virginia: 2014

Suggested citation:
Baker, E.J., and Kellogg, C.A., 2014, Composition of three DNA extraction kits to establish maximum yield and quality of coral-associated microbial DNA: U.S. Geological Survey Open-File Report 2014-1066, 14 p., *http://dx.doi.org/10.3133/ofr20141066*.

ISSN 2331-1258 (online)

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Abstract

Coral microbiology is an expanding field, yet there is no standard DNA extraction protocol. Although many researchers depend on commercial extraction kits, no specific kit has been optimized for use with coral samples. Both soil and plant DNA extraction kits from MO BIO Laboratories, Inc. have been used by many research groups for this purpose. MO BIO recently replaced their PowerPlant® kit with an improved PowerPlantPro kit, but it was unclear how these changes would affect the kit's use with coral samples. In order to determine which kit produced the best results, we conducted a comparison between the original PowerPlant kit, the new PowerPlantPro kit, and an alternative kit, PowerSoil, using samples from several different coral genera. The PowerPlantPro kit had the highest DNA yields, but the lack of 16S rRNA gene amplification in many samples suggests that much of the yield may be coral DNA rather than microbial DNA. The most consistent positive amplifications came from the PowerSoil kit.

Introduction

Due to increased reports of coral diseases and rising interest in the associated microbiomes of animals, coral microbiology has grown and diversified. Researchers seek to characterize the coral holobiont (the totality of the coral animal plus microbial symbionts) in order to explore dispersal, biodiversity, disease, and other aspects of fragile reef ecosystems. Since 2001 molecular techniques such as DNA extraction followed by DNA sequencing have been employed to assess microbial biodiversity and richness in corals (Rohwer and others, 2001). Technical advances, such as pyrosequencing (also known as 'second-generation' or 'next-generation' sequencing), have driven manufacturers of biological kits and reagents to optimize and specialize their DNA extraction products to improve the overall quality of recoverable data from environmental samples. In spite of the increase in coral microbiology research and publication over the past 12 years (for example, Rohwer and others, 2001; Kellogg, 2004; Bourne and Munn, 2005; Lampert and others, 2008; Kellogg and others, 2009; Lins-de-Barros and others, 2010; Kellogg and others, 2013), we are unaware of any commercial kit optimized for the extraction of microbial DNA from coral samples. Kits designed for other sample types have been used 'off-label' for corals, to include soil kits (Rohwer and others, 2001; Penn and others, 2006; Kellogg and others, 2009; Reis and others, 2009; Godwin and others, 2012; Santos and others, 2012), plant kits (Sunagawa and others, 2009; Sato and others, 2010; Sunagawa and others, 2010; Santos and others, 2012; Bayer and others, 2013; Roder and others, 2014) and blood and tissue kits (Sweet and others, 2011).

In 2010, Sunagawa and others published a popular protocol based on a modified version of the MO BIO PowerPlant DNA Isolation Kit. Modifications included the addition of chemical lysis agents (lysozyme and protease) and smaller zirconia/silica beads to break open microbial cells during physical lysis. This protocol has been used in a number of recent studies (Sunagawa and others, 2010; Kellogg and others, 2012; Bayer and others, 2013; Kellogg and others, 2013). In early 2013, MO BIO discontinued production of the PowerPlant kit and replaced it with a new product, the PowerPlantPro kit. The

kit's introduction necessitated an evaluation of its compatibility with the Sunagawa protocol and a determination of the effectiveness of its extraction results with coral samples when compared to the original PowerPlant kit. Furthermore, the PowerSoil kit had been previously used with coral samples (Barneah and others, 2007; Kellogg and others, 2009; Chen and others, 2011; Godwin and others, 2012), so at the manufacturer's suggestion, both plant-specific kits were compared to the MO BIO PowerSoil kit.

The objective of this study was to evaluate the effectiveness of three MO BIO DNA extraction kits (PowerPlant, PowerPlantPro, PowerSoil) with each kit incorporating the modifications outlined by Sunagawa and others (2010). Effectiveness was based on positive 16S rRNA gene amplification, using the extracted samples as templates, and by using PicoGreen® to quantify the total DNA yield. The experiment used four coral species: *Orbicella annularis*, *Siderstrea siderea*, *Lophelia pertusa,* and *Primnoa resedaeformis*. The chosen coral species vary with respect to morphology, symbiont presence and preservation method, all of which can contribute to differences in extraction efficiency (table 1).

Table 1. Coral sample characteristics and taxonomy.

Genus/Species	Order	Family	Photosynthetic Symbionts	Preservation Method
Orbicella annularis	Scleractinia	Faviidae	Yes	Liquid Nitrogen
Siderastrea siderea	Scleractinia	Siderastreidae	Yes	Liquid Nitrogen
Primnoa resedaeformis	Alcyonacea	Primnoidae	No	RNAlater
Lophelia pertusa	Scleractinia	Caryophylliidae	No	RNAlater

Materials and Methods

Biological replicates (n = 5) from different colonies of four coral species were used for microbial DNA extractions. For each biological replicate, duplicate extractions were conducted by placing 50 milligrams (mg) of coral sample into the 2 millilitre (mL) bead tubes supplied with each kit. Samples preserved in liquid nitrogen (*O. annularis* and *S. siderea*) were ground to powder on dry ice and consisted of a slurry of coral mucus, tissue, and skeleton. Samples preserved in RNAlater® (*P. resedaeformis* and *L. pertusa*) were processed as coral tissue with associated mucus. *Primnoa resedaeformis* samples were collected by excising two polyps (50 mg) from a coral branch with flame-sterilized forceps. *Lophelia pertusa* fragments were placed in sterile aluminum weigh boats, and one or two calyxes were cracked open with a flame-sterilized hammer. Mucus and tissue were then separated from the calcium carbonate skeleton with an airbrush and sterile phosphate-buffered saline. Care was taken to ensure that no skeletal fragments were collected with the resultant slurry of mucus and tissue.

Three types of MO BIO DNA isolation kits were tested: the newly specialized MO BIO PowerPlantPro DNA Isolation Kit (catalog no. 13400-50 MO BIO Laboratories; Carlsbad, Calif.), the original MO BIO PowerPlant DNA Isolation Kit (catalog no. 13200-100 MO BIO Laboratories, Carlsbad, Calif.), and the PowerSoil DNA Isolation Kit (catalog no. 12888-100 MO BIO Laboratories, Carlsbad, Calif.). Each kit was modified (Sunagawa and others, 2010) as shown in the detailed protocols supplied in Appendices 1–3. The first modification was the addition of 0.19 microlitres (µL) Ready-Lyse™ Lysozyme Solution (catalog no. R1810M Epicentre; Madison, Wis.) to the manufacturer provided bead solution/sample mix coupled with a 10 minute room temperature incubation period. The second alteration was the addition of 25 µL of Proteinase K Solution (catalog no. AM2546 Ambion®; Foster City, Calif.) to the lysozyme/bead solution mixture; this was followed by an incubation period at 65 °C for 60 minutes. Finally, 400 mg each of sterile 0.1 and 0.5 millimeter (mm) zirconia/silica beads (catalog nos. 11079105z and 11079101z BioSpec Products; Bartlesville, Okla.) were added to the samples. Samples were disrupted using a Mini-Beadbeater-1 (catalog no. 3110BX Biospec Products,

Inc.; Bartlesville, Okla.) for 30 seconds each instead of the typical 10 minute vortexing step. Post-homogenization protocol steps reverted to those outlined by the manufacturer for each individual kit. (Step-wise protocol for each kit can be found in Appendices 1–3). Two replicate DNA extractions were performed for each coral sample (20 samples; 40 extractions total) and duplicate extractions were pooled to produce 60 µL samples for use in downstream applications.

Extractions were used as templates for amplification by polymerase chain reaction (PCR) on a GeneAmp PCR system (Applied Biosystems), using 12.5 µL AmpliTaq Gold 360 Master Mix (catalog no. 4398881 AB Applied Biosystems; Bartlesville, Okla.), 1 µL each of 10 picomolar (pM) concentrations of forward 63F (Marchesi and others, 1998)(5'CAGGCCTAACACATGCAAGTC3') (IDT; Iowa City, Iowa) and reverse primers 1542R (Pantos and others, 2003) (5'AAGGAGGTGATCCAGCCGCA3') (IDT; Iowa City IA), 0.5 µL of sterile deionized water (catalog no. AM9937 Ambion; Foster City, Calif.), and 10 µL DNA template, for a total volume of 25 µL. The Bourne and Munn (2005) thermocycler program was used : initial cycle of 95 °C for 15 minutes; 30 subsequent cycles of 95 °C for 1 minute each, followed by a 54 °C cycle for 1 minute, a 72 °C for 2 minutes; and a final extension of 72 °C for 10 minutes. PCR products were verified on a 1 percent agarose gel stained with ethidium bromide at a concentration of 10^{-7} grams per millilitre (g/mL) by means of electrophoresis at 60 volts for 1.5 hours.

DNA concentrations of extracts were measured by using Quant-iT™ PicroGreen dsDNA Assay Kit (catalog no. P7589 Invitrogen: Eugene, Oreg.) in conjunction with a mini spectral fluorometer (Model no. 8000-003 Turner Designs: Sunnyvale, Calif.) as outlined in the manufacturer's protocol.

Results & Discussion

The aim of this study was to compare three different MO BIO DNA extraction kits in order to assess the quantity and quality of the microbial DNA output from coral samples. A total of 120 extractions from four coral species were performed with three different MO BIO extraction kits: the original PowerPlant kit, the updated PowerPlantPro kit, and the PowerSoil kit. Each coral holobiont is unique, so various corals were examined to assess factors that could affect extraction efficiency, such as physiology, symbiont presence, and preservation method.

Obtaining quality microbial DNA extractions from corals has proven challenging for many research groups (Pollock and others, 2011; Santos and others, 2012). Simultaneous extraction of coral host DNA alongside eukaryotic microbe DNA, such as zooxanthellae or fungi, can overwhelm the prokaryotic microbial component. In addition to challenges arising from environmental sample heterogeneity, high levels of variability—with respect to bacterial diversity of cell wall compositions and cellular structures—can affect microbial DNA extraction efficiency.

The most widely used and highly conserved bacterial gene sequence, which primers have been optimized to selectively amplify, is the 16S rRNA gene sequence (Amann and others, 1995). Primers 63F and 1542R were chosen to amplify bacterial 16S rRNA genes from coral samples in this experiment in place of the widely used 8F (also known as 27F) (Edwards and others, 1989) and 1492R (Stackebrandt and Liesack, 1993) primers. The reason is that the "bacterial-specific" primer 8F has also been found to amplify coral 18S rRNA genes from some coral samples (Galkiewicz and Kellogg, 2008). This sequence homology issue, which made it difficult to distinguish between the presence of bacterial or coral DNA amplification, was circumvented by using 63F and 1542R primers (Galkiewicz and Kellogg, 2008; Littman and others, 2009). These primers result in bacterial amplicons of approximately 1500 base pairs (bp) and coral amplicons (if any) of approximately 600-700 bp. This distinction

between coral and bacterial amplicons allowed us to better evaluate the efficacy of these kits for microbial DNA extraction.

All three kits had minimal success extracting microbial DNA from *Orbicella annularis* samples preserved in liquid nitrogen. This was an unexpected outcome since the Sunagawa modifications were developed using samples of *O. faveolata* collected and preserved in an identical fashion (Sunagawa and others, 2009). The most successful coral, in terms of positive 16S rRNA amplification across all three MO BIO kits, was *S. siderea*, which was also preserved in liquid nitrogen. However, *S. siderea* had an unexpected amplification of multiple bands regardless of extraction kit. We had not observed this outcome with any other coral sample, but suspect that these additional bands are due to unspecific binding of this particular primer set. Since all kits performed on similar levels with respect to *O. annularis* and *S. siderea* (figs. 1 & 2), it would seem that the choice of extraction kit had less influence on the outcome than the choice of coral host, at least for samples preserved in liquid nitrogen. This finding suggests that the results of extraction kit comparisons that used single coral species (Santos and others, 2012) may not correlate well with tests that used differing coral species. It is possible that some of the variability in results with these two coral species is due to the presence of calcium carbonate skeleton in the samples, as the entire frozen biopsy of mucus, tissue, and skeleton is ground together into a single powder, even though efforts were made to minimize the amount of skeletal inclusion. For the cold-water corals, *L. pertusa* and *P. resedaeformis*, it was possible to separate the mucus and tissue from the skeleton completely before extraction due to their large polyp size.

Extractions using the original MO BIO PowerPlant kit resulted in appropriately sized 1500 bp bands in all four of the different coral samples (figs. 1, 2, 3, and 4). The amplicons from *L. pertusa* were

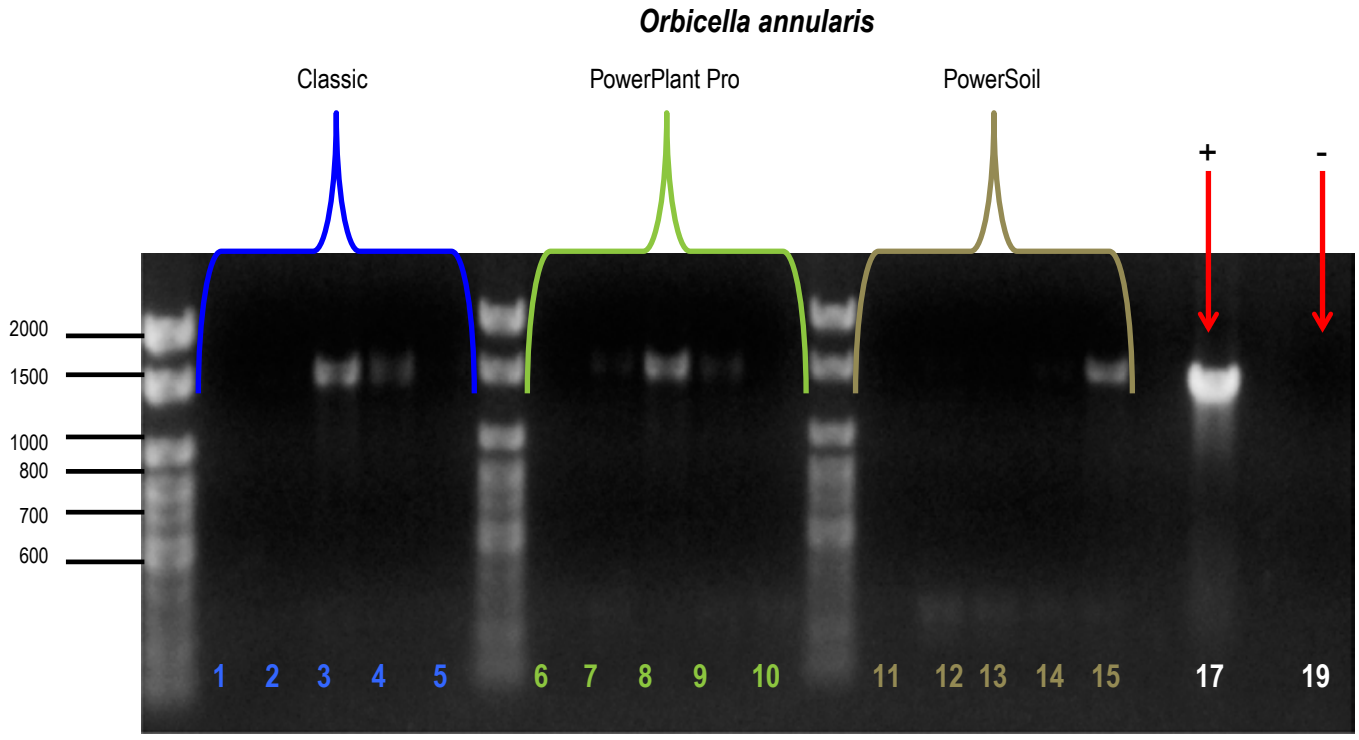

Figure 1. *Orbicella annularis* electrophoresis gel: lanes 1–5 are amplifications based on extractions using the original PowerPlant kit with Sunagawa modifications, lanes 6–10 are amplifications based on extractions using the new PowerPlant Pro kit with integrated Sunagawa protocol, and lanes 11–15 are amplifications based on extractions using the PowerSoil kit with integrated Sunagawa modifications. Lane 17 = positive control. Lane 19 = negative control.

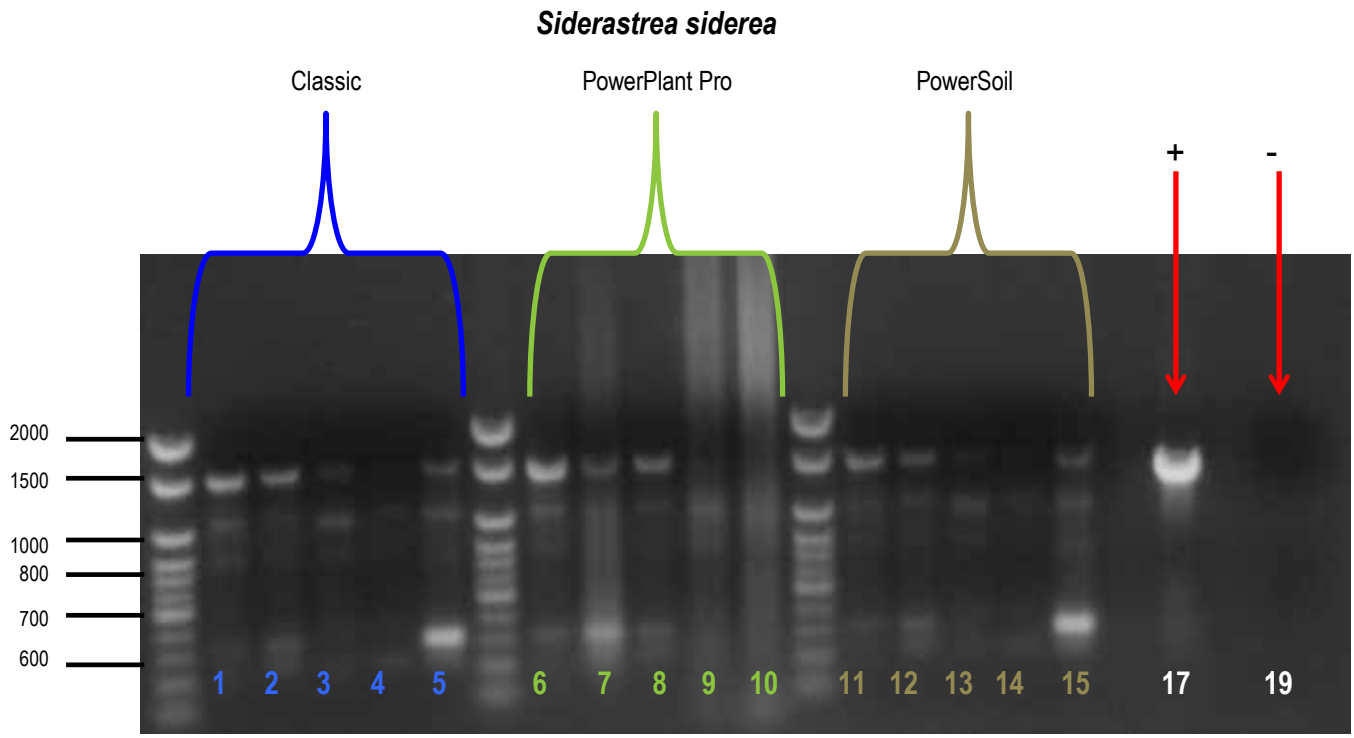

Figure 2. *Siderastrea siderea* electrophoresis gel: lanes as described in figure 1.

faint when using the original PowerPlant kit with Sunagawa modifications (fig. 4). In terms of consistency, extractions using the original PowerPlant kit produced positive 16S rRNA amplicons in approximately 45 percent of the experimental samples, performing best with *S. siderea* (table 2). The original MO BIO PowerPlant kit with Sunagawa modifications yielded the lowest total DNA concentrations of the three MO BIO kits with all coral samples except for *L. pertusa* (fig. 5). This poor performance was unexpected since this protocol was the comparison standard and has been used successfully with both scleractinian and gorgonian corals (Sunagawa and others, 2010; Bayer and others, 2013; Kellogg and others, 2013).

Amplification using templates from the PowerPlantPro MO BIO DNA extraction kit with Sunagawa modifications produced appropriately sized 1500bp bands in some *O. annularis* and *S. siderea* samples, but failed to produce bands in *L. pertusa* or *P. resedaeformis* (figs. 1, 2, 3, and 4). The PowerPlantPro kit was the least consistent kit, with positive 16S rRNA gene amplification in only 35 percent of the experimental samples (table 2). The new MO BIO PowerPlantPro kit yielded the highest concentrations of total DNA (fig. 5). The high total DNA concentrations coupled with the low rate of successful bacterial amplification suggests that the PowerPlantPro did a superior job extracting eukaryotic DNA from the coral host when compared to the PowerSoil and original PowerPlant DNA extraction kits. Given that the few positive amplifications were from the zooxanthellate coral samples, it is possible that the kit preferentially extracted DNA from the algal symbionts and that the amplicons resulted from their chloroplast 16S rRNAs. Zooxanthellate and azooxanthellate corals were deliberately included in this experiment (table 1) to assess whether plant-optimized extraction kits behave differently when extracting from photosynthetic corals versus those lacking algal symbionts. Finally, it is also possible that the PowerPlantPro kit permitted carryover of more inhibitors than the other kits.

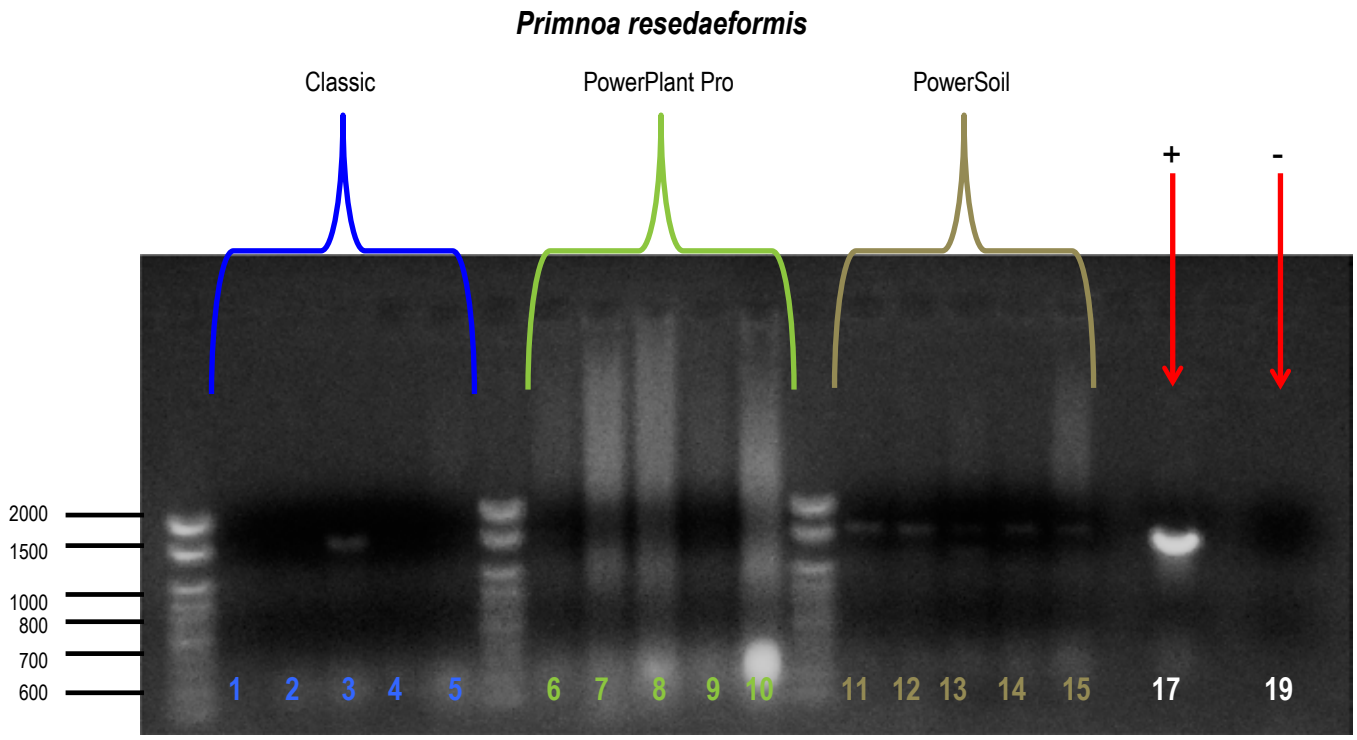

Figure 3. *Primnoa resedaeformis* electrophoresis gel: lanes as described in figure 1.

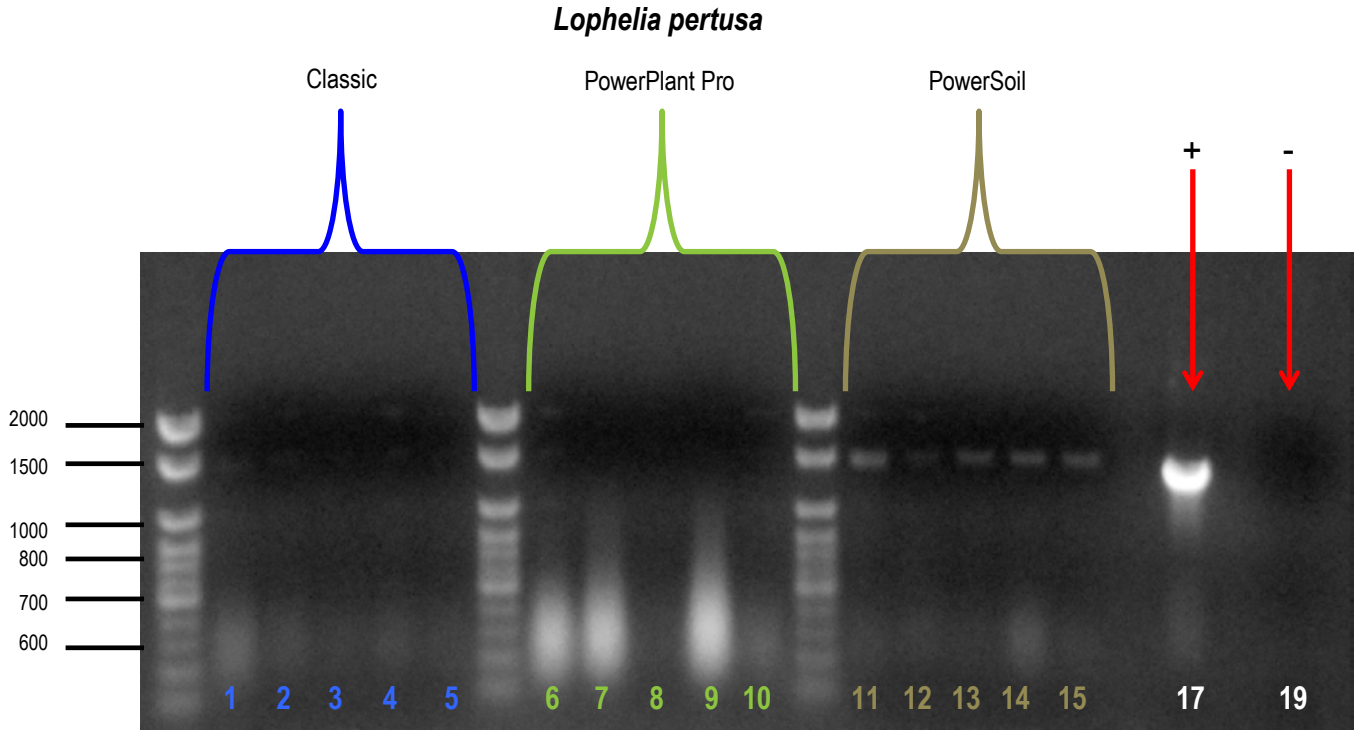

Figure 4. *Lophelia pertusa* electrophoresis gel: lanes as described in figure 1.

Table 2. Successful extractions designated on a per coral, per kit basis

	Orbicella annularis	*Siderastrea siderea*	*Primnoa resedaeformis*	*Lophelia pertusa*	Average Success
Original PowerPlant	2 of 5 (40%)	4 of 5 (80%)	1 of 5 (20%)	2 of 5 (40%)	45%
PowerPlant Pro	3 of 5 (60%)	3 of 5 (60%)	0 of 5 (0%)	1 of 5 (20%)	35%
PowerSoil	1 of 5 (20%)	4 of 5 (80%)	5 of 5 (100%)	5 of 5 (100%)	75%

The MO BIO PowerSoil DNA extraction kit with Sunagawa modifications was the most successful at extracting microbial DNA from a variety of corals. With the exception of *O. annularis*, this kit worked well regardless of coral type (hard or soft) or preservation method, producing positive 16S rRNA gene amplicons in all corals tested (figs. 1, 2, 3, and 4). The PowerSoil kit was the best of the MO BIO kits tested in terms of consistency, showing positive 16S rRNA amplification in approximately 75 percent of the experimental samples tested (table 2). It was particularly effective on the cold-water corals, with amplification in 100 percent of the samples (figs. 3 and 4). It is unclear whether this result might be due to the preservation method (RNAlater) or to the lack of coral skeleton in the samples. The total DNA concentrations from the PowerSoil kit were highly variable among the different coral samples, particularly with *S. siderea* (fig. 5). Similar tests using the coral *Mussismilia harttii* (Santos and others, 2012) found PowerSoil (without Sunagawa modifications) to be consistent across three replicates in terms of DNA yield; however, as the data show, different holobionts result in different extraction efficiencies. The overall high level of consistency observed in extracting verifiable bacterial DNA, coupled with total DNA concentration values markedly higher than the original PowerPlant kit, made the PowerSoil kit with Sunagawa modifications the best kit tested for examining microbes associated with corals.

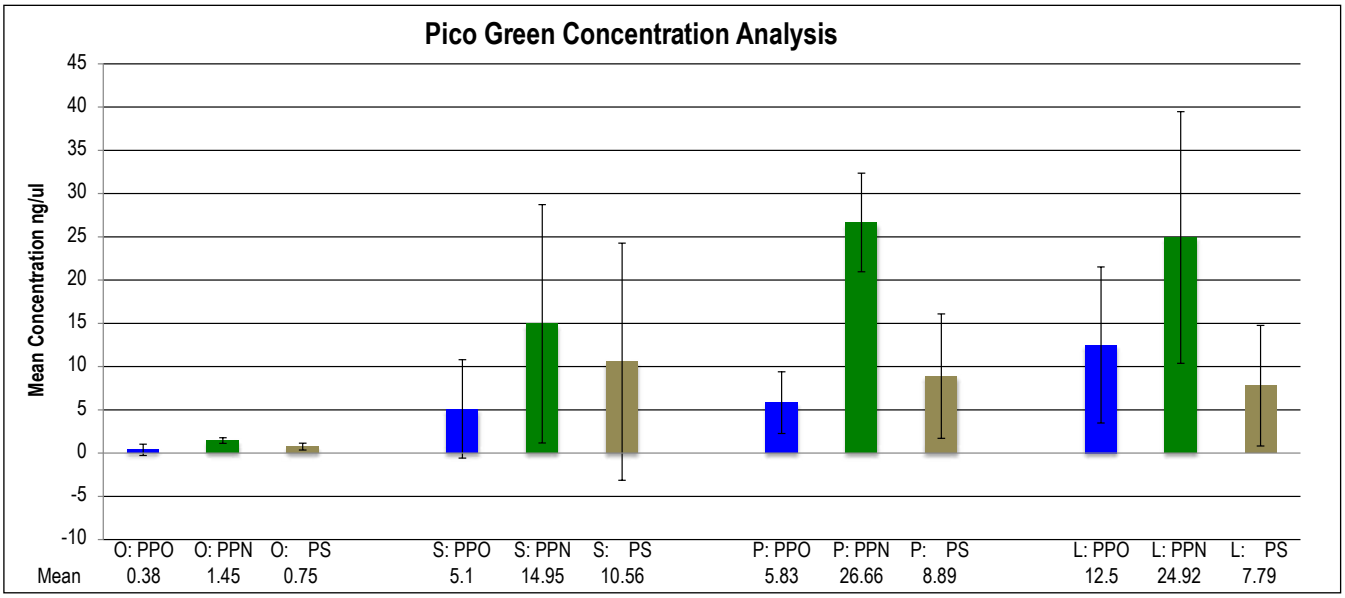

Figure 5. Graphical depiction of mean DNA concentrations in sample extractions. O = *Orbicella annularis*. S = *Siderastrea siderea*, P = *Primnoa resedaeformis*, L = *Lophelia pertusa*, PPO (blue) = the use of the original MOBIO PowerPlant protocol with Sunagawa modifications, PPN (green) = the use of the MOBIO PowerPlant Pro DNA extraction kit with Sunagawa modifications, PS (brown) = the use of the MOBIO PowerSoil DNA extraction kit. The error bars represent the standard deviation observed in each n=5 sample set.

While the methodology underlying all three of the MO BIO extraction kits is similar, our results show that the kits are not equivalent for use in coral microbial ecology. A caveat is that successful amplification of the same sample from different kits (for example, PowerPlant versus PowerSoil) may also yield different bacterial diversity due to inherent biases within each extraction (see results in Santos and others, 2012). Based on the broad and robust performance of the Sunagawa-modified PowerSoil kit, we advocate its use for coral samples, realizing that the results, while similar, may not be identical to previous work conducted on extractions using the modified PowerPlant kit.

Acknowledgments

We thank MO BIO for providing us with the PowerPlantPro kit to test.

These samples of *Orbicella annularis* were collected under permit DRTO-2009-SCI-0018 (study DRTO-00074) from the Dry Tortugas National Park, and samples of *Siderastrea siderea* were collected under permit VIIS-2008-SCI-0033 (study VIIS-08033) from the Virgin Islands National Park.

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Appendix 1

The Sunagawa protocol (which is based on the original MO BIO PowerPlant® DNA Isolation Kit.) (Sunagawa and others, 2010)

[mg, milligram; g, gram; µL, microlitre; mL, millilitre; mm, millimeter; °C, degrees Celsius; rpm, revolutions per minute; X g, relative centrifugal force (i.e., 500 X g is 500 times the force of gravity)]

Sunagawa Protocol Using Original PowerPlant

Protocol Step	Description	Notes
1	Prep Sample	
2	Transfer approximately 50 mg (0.05 g) to a PowerPlant bead tube and add 550 µL of PowerPlant Bead Solution.	Unaltered from original PowerPlant protocol step 1.
3	Add 0.19 µL of Ready-Lyse Lysozyme Solution, vortex briefly and incubate for 10 minutes at room temperature.	Step added per Sunagawa protocol.
4	Add 60 µL of solution PB1, and 25 µL of Proteinase K (20 mg/mL), and vortex briefly.	The addition of proteinase K along with vortexing to mix was added by the Sunagawa protocol, the addition of 60 µL of PB1 corresponds to step 4 of the original PowerPlant protocol.
5	Incubate for 60 minutes at 65 °C in hybridization oven or water bath.	Sunagawa protocol increased the incubation time from 10 minutes to 60 minutes from step 5 of the original PowerPlant protocol.
6	Add 400 mg (0.40 g) of each 0.1 mm zirconia/silica beads and 0.5 mm zirconia/silica beads and bead beat for 30 seconds using a Mini-Beadbeater-1 at 5,000 rpm.	Homogenization via the beadbeater with additional silica beads was added per the Sunagawa Protocol. This substitutes the vortexing step 6 of the original PowerPlant protocol.
7	Centrifuge for 2 minutes at 10,000 X g at room temperature, and transfer supernatant to new 1.5 mL collection tube by decanting.	Sunagawa increased the duration of centrifugation from 30 seconds to 2 minutes. In addition the size of the centrifuge tube was decreased from 2 mL to 1.5 mL, and the use of decanting as a means of collecting the supernatant were outlined in the Sunagawa protocol.
8	Centrifuge for 2 minutes at maximum speed of centrifuge (typically 16,000 X g) at room temperature and transfer 250 µL of the supernatant to a 2 mL collection tube.	Step added per Sunagawa protocol.
9	Add 250 µL of solution PB2, mix, and incubate for 5 minutes at 4 °C.	This step was unaltered from the original PowerPlant protocol step 9
10	Centrifuge for 5 minutes at 23,000 X g (15,000 rpm) at 4 °C and quickly transfer supernatant to new 1.5 mL collection tube (work in order)	This entire step was added in the Sunagawa protocol.
11	Centrifuge for 5 minutes at 23,000 X g (15,000 rpm) at 4° C and quickly transfer supernatant to new 1.5 mL collection tube (work in reverse this time)	This entire step was added in the Sunagawa protocol.
12	Add 500 µL of solution PB3, mix and incubate for 10 minutes at room temperature.	The amount of solution was decreased from 1 mL to 500 µL as stated in step 12 of the original PowerPlant protocol.
13	Centrifuge for 20 minutes at 23,000 X g at 4 °C.	Centrifugation was altered by increasing the duration, from 15 minutes to 20 minutes increasing the speed from 13,000 X g to 23,000 X g, and specifying a temperature of 4 °C. This step corresponds to step 13 of the original PowerPlant protocol.
14	Remove supernatant and resuspend pellet in 100 µL of solution PB6.	This step was unaltered from the original PowerPlant protocol step 14
15	Add 500 µL of PB4, mix and transfer too spin column.	This step was unaltered from the original PowerPlant protocol step 15
16	Centrifuge for 1 minute at 10,000 X g at room temperature	This step was unaltered from the original PowerPlant protocol step 16
17	Discard flow through, and add 500 µL of PB5	This step was unaltered from the original PowerPlant protocol step 18
18	Centrifuge for 30 seconds at 10,000 X g at room temperature.	This step was unaltered from the original PowerPlant protocol step 18

Protocol Step	Description	Notes
19	Transfer spin filter to a new 1.5 mL centrifuge tube.	A new 1.5 mL centrifuge tube is used here instead of discarding the flow through as the original PowerPlant protocol stated in step 19.
20	Centrifuge for 1 minute at 16,000 X g at room temperature. Transfer spin filter to new 1.5 mL collection tube	A 1.5 mL centrifuge tube is used instead of a 2 mL as stated in step 21 of the original PowerPlant protocol.
21	Add 30 µL of solution PB6 to center of spin filter, and let sit at room temperature for 1 minute.	The amount of solution PB6 that is utilized is decreased from 50 µL to 30 µL.
22	Elute DNA by centrifugation 1 minute at 16,000 X g at room temperature.	The speed centrifugation is increased from 10,000 X g to 16,000 X g and duration of centrifugation is increased from 30 seconds to 1 minute. This corresponds to step 23 in the original PowerPlant protocol.

Appendix 2

The new MO BIO PowerPlant® Pro DNA Isolation Kit protocol adapted to include the Sunagawa modifications.

[mg, milligram; g, gram; µL, microlitre; mL, millilitre; mm, millimeter; °C, degrees Celsius; rpm, revolutions per minute; X g, relative centrifugal force (i.e., 500 X g is 500 times the force of gravity)]

Sunagawa Protocol Using New PowerPlant Pro DNA Isolation Kit

Protocol Step	Description	Notes
1	Prep Sample	
2	Transfer approximately 50 mg (0.05 g) to a power plant bead tube and add 450 µL of PD1.	Unaltered from PowerPlant Pro protocol step 1.
3	Add 0.19 µL of Ready-Lyse Lysozyme Solution, vortex briefly and incubate for 10 minutes at room temperature.	Step added per Sunagawa protocol.
4	Add 50 µL of solution PD2, and 25 µL of Proteinase K (20 mg/mL), and vortex briefly.	The addition of proteinase K along with vortexing to mix was added in accordance with the Sunagawa protocol, the addition of 50 µL of PD2 corresponds to step 2 of the PowerPlant Pro protocol.
5	Incubate for 60 minutes at 65 °C in hybridization oven or water bath.	This step was added in order to incorporate the Sunagawa protocol.
6	Add 3 µL of RNase solution vortex briefly and incubate at room temperature for 5 minutes.	This step corresponds to step 3 in the new PowerPlant Pro.
7	Add 400 mg (0.40 g) of each 0.1 mm zirconia/silica beads and 0.5 mm zirconia/silica beads and bead beat for 30 seconds using a Mini-Bead Beater-1 at 5,000 rpm.	Homogenization via the bead beater with additional silica beads was added per the Sunagawa Protocol. This replaces the vortexing and homogenization steps described in step 4 of the PowerPlant Pro protocol.
8	Centrifuge for 2 minutes at 13,000 X g at room temperature, and transfer supernatant to new 1.5 mL collection tube by decanting.	This step should occur at room temperature and the size of the centrifuge tube was decreased from 2 mL to 1.5 mL, as outlined in the Sunagawa protocol. This step corresponds with steps 5 & 6 of the manufacture's protocol.
9	Centrifuge for 2 minutes at maximum speed of centrifuge (typically 16,000 X g) at room temperature and transfer 250 µL of the supernatant to a 2 mL collection tube.	Step added per Sunagawa protocol.
10	Add 250 µL of solution PD3, mix, and incubate for 5 minutes at 4 °C.	This step was unaltered from the PowerPlant Pro protocol step 7.
11	Centrifuge for 5 minutes at 23,000 X g (15,000rpm) at 4 °C and quickly transfer supernatant to new 1.5 mL collection tube (work in order).	This entire step was added in the Sunagawa protocol.

Protocol Step	Description	Notes
12	Centrifuge for 5 minutes at 23,000 X g (15,000 rpm) at 4 °C and quickly transfer supernatant to new 1.5 mL collection tube (work in reverse this time).	This step was added per the Sunagawa protocol.
13	Avoiding the pellet transfer up to 600 µL of the supernatant to a clean 2 mL collection tube.	This step corresponds to step 9 of the PowerPlant Pro protocol.
14	Add 600 µL of PD4 and 600 µL of PD6 vortex to mix for 5 seconds.	This step corresponds to step 10 of the PowerPlant Pro protocol.
15	Load ~600 µL of the lysate onto the spin filter and centrifuge at 10,000 X g for 30 seconds. Discard flow through. Replace filter and add another 600 µL of lysate to the spin filter and centrifuge at 10,000 X g for 30 seconds. Discard the flow through and repeat a third time until all of the lysate has been passed through the spin filter. Discard flow through and replace spin filer in the collection tube.	This step corresponds to step 11 of the PowerPlant Pro protocol.
16	Add 500 µL of Solution PD5 to the spin filter column. Centrifuge for 30 seconds at 10,000 X g. Discard flow through. Place spin filter into the same collection tube.	This step corresponds to step 12 of the PowerPlant Pro protocol.
17	Add 500 µL of solution PD6 to the spin filter column. Centrifuge for 30 seconds at 10,000 X g. Discard flow through. Place spin filter back in the same collection tube.	This step corresponds to step 13 of the PowerPlant Pro protocol.
18	Centrifuge for 2 minutes at up to 16,000 X g to remove residual PD6 solution.	This step corresponds to step 14 of the PowerPlant Pro protocol.
19	Carefully place the spin filter into a new clean 2 mL collection tube.	This step corresponds to step 15 of the PowerPlant Pro protocol.
20	Add 30 µL of PD7 to the center of the white filter membrane and incubate at room temperature for 2 minutes.	The amount of solution PB6 that is utilized is decreased from 50 µL to 30 µL in order to be consistent with volumes in the Sunagawa protocol. This corresponds to step 16 in the PowerPlant Pro protocol.
21	Centrifuge for 1 minute at 16,000 X g.	As outlined by Sunagawa the speed of centrifugation is increased from 10,000 X g to 16,000 X g and duration of centrifugation is increased from 30 seconds to 1 minute. This corresponds to step 17 in the PowerPlant Pro protocol.
22	Discard spin filter, and store DNA at -20 °C.	This step corresponds to step 18 of the PowerPlant Pro protocol.

Appendix 3

The new MO BIO PowerSoil® DNA Isolation Kit protocol adapted to include the Sunagawa modifications.

[mg, milligram; g, gram; µL, microlitre; mL, millilitre; mm, millimeter; °C, degrees Celsius; rpm, revolutions per minute; X g, relative centrifugal force (i.e., 500 X g is 500 times the force of gravity)]

Sunagawa Protocol Using PowerSoil DNA Isolation Kit

Protocol Step	Description	Notes
1	Prep Sample	
2	Transfer approximately 50 mg (0.05 g) of sample to a PowerSoil bead tube comes preloaded with buffer and fragments of garnet.	The amount of sample utilized was decreased from 0.25 g to 0.05 g in order to remain consistent with sample size outlined in the Sunagawa protocol.
3	Add 0.19 µL of Ready-Lyse Lysozyme Solution, vortex briefly and incubate for 10 minutes at room temperature.	Step added per Sunagawa protocol.
4	Add 60 µL of solution C1, and 25 µL of Proteinase K (20 mg/mL), and vortex briefly.	The addition of proteinase K along with vortexing to mix was added in accordance with the Sunagawa protocol, the addition of 60 µL of C1 corresponds to step 4 of the PowerSoil protocol.

Protocol Step	Description	Notes
5	Incubate for 60 minutes at 65 °C in hybridization oven or water bath.	This step was added in order to incorporate the Sunagawa protocol.
6	Add 400 mg (0.40 g) of each 0.1 mm zirconia/silica beads and 0.5 mm zirconia/silica beads and bead beat for 30 seconds using a Mini-Bead Beater-1 at 5,000 rpm.	Homogenization via the bead beater with additional silica beads was added per the Sunagawa Protocol. This substitutes the vortexing steps described in step 5 of the PowerSoil protocol.
7	Centrifuge for 2 minutes at 10,000 X g at room temperature, and transfer supernatant to new 1.5 mL collection tube by decanting.	The duration of centrifugation was increased from 30 seconds to 2 minutes and the size of collection tube was decreased from 2.0 mL to 1.5 mL per the Sunagawa protocol. This corresponds with steps 6 and 7 in the manufacturer's protocol.
8	Centrifuge for 2 minutes at maximum speed of centrifuge (typically 16,000 X g) at room temperature and transfer 250 µL of the supernatant to a 2 mL collection tube.	Step added per Sunagawa protocol.
9	Add 250 µL of solution C2, mix, and incubate for 5 minutes at 4 °C.	This step was unaltered from the PowerSoil protocol step 8.
10	Centrifuge for 5 minutes at 23,000 X g (15,000 rpm) at 4 °C and quickly transfer supernatant to new 1.5 mL collection tube (work in order).	This entire step was added as outlined in the Sunagawa protocol.
11	Centrifuge for 5 minutes at 23,000 X g (15,000 rpm) at 4 °C and quickly transfer supernatant to new 1.5 mL collection tube (work in reverse this time).	This entire step was added as outlined in the Sunagawa protocol.
12	Avoiding the pellet transfer up to 600 µL of the supernatant to a clean 2 mL collection tube.	This step corresponds to step 10 of the PowerSoil protocol.
13	Add 200 µL of C3 vortex briefly incubate at 4 °C for 5 minutes.	This step corresponds to step 11 of the PowerSoil protocol.
14	Centrifuge tubes at room temperature for 1 minute at 10,000 X g.	This step corresponds to step 12 of the PowerSoil protocol.
15	Avoiding the pellet transfer up to, but no more than 750 µL of supernatant to a clean 2.0 mL centrifuge tube.	This step corresponds to step 13 of the PowerSoil protocol.
16	Shake to mix solution C4 before use. Add 1200 µL of solution C4 to the supernatant and vortex for 5 seconds.	This step corresponds to step 14 of the PowerSoil protocol.
17	Load ~675 µL of the lysate onto the spin filter and centrifuge at 10,000 X g for 1 minute at room temperature. Discard flow through. Replace filter and add another 675 µL of lysate to the spin filter and centrifuge at 10,000 X g for 1 minute at room temperature. Discard the flow through and repeat a third time till all of the lysate has been passed through the spin filter. Discard flow through and replace spin filter in the collection tube.	This step corresponds to step 15 of the PowerSoil protocol.
18	Add 500 µL of Solution C5 to the spin filter column. Centrifuge for 30 seconds at 10,000 X g. Discard flow through.	This step corresponds to steps 16 & 17 of the PowerSoil protocol.
19	Centrifuge again at room temperature for 1 minute at 16,000 X g.	This step corresponds to step 18 of the PowerSoil protocol.
20	Place spin filter into clean 1.5 mL collection tube	This step corresponds to step 19 of the PowerSoil protocol. The collection tube size is decreased from 2.0 to 1.5 mL as outlined in the Sunagawa protocol.
21	Add 30 µL of C6 to the center of the white filter membrane and incubate at room temperature for 2 minutes	The amount of solution C6 that is utilized is decreased from 100 µL to 30 µL in order to be consistent with volumes in the Sunagawa protocol. This corresponds to step 20 in the PowerSoil protocol.
22	Centrifuge for 1 minute at 16,000 X g.	As outlined by Sunagawa the speed of centrifugation is increased from 10,000 X g to 16,000 X g and duration of centrifugation is increased from 30 seconds to 1 minute. This corresponds to step 17 in the PowerSoil protocol.

ISSN 2331-1258 (online)
http://dx.doi.org/10.3133/ofr20141066