Multi-macromolecular extraction from endosymbiotic anthozoans

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Abstract

Obligately symbiotic associations between reef-building corals (anthozoan cnidarians) and photosynthetically active dinoflagellates of the family Symbiodiniaceae comprise the functional basis of all coral reef ecosystems. Given the existential threats of global climate change to these generally thermo-sensitive entities, there is an urgent need to better understand the physiological implications of changes in the abiotic milieu of scleractinian corals and their mutualistic algal endosymbionts. Although initially slow to leverage the immense breakthroughs in molecular biotechnology that have benefited humankind, coral biologists are making up for lost time in exploiting an array of ever-advancing molecular tools for answering key questions pertaining to the survival of corals in an ever-changing world. In order to comprehensively characterize the "multi-'Omic" landscape of the coral "holobiont-" the cnidarian host, its intracellular dinoflagellates, and a plethora of other microbial constituents- I introduce a series of protocols herein that yield large quantities of high-quality RNA, DNA, protein, lipids, and polar metabolites from a diverse array of reef corals and endosymbiotic sea anemones (which are often used as model organisms for processes that are difficult to study in corals). Although numerous published articles in the invertebrate zoology field feature protocols that lead to sufficiently high yield of intact host coral macromolecules, through using the approach outlined herein one may simultaneously acquire a rich, multi-compartmental pool of macromolecules that truly reflects the complex and dynamic nature of these animal-plant chimeras.

Safety considerations

The extraction of high quantities of RNAs, DNAs, proteins, lipids, and polar metabolites warrants the use of toxic, corrosive chemicals, namely an acid-guanidinium+ phenol-based solvent (e.g., TRIzol®, Thermo-Fisher Scientific [TFS]) and chloroform. All work should be undertaken while wearing nitrile gloves, a laboratory coat, safety goggles, and closed-toe shoes. All steps should be undertaken in a fume hood except for those associated with the RNA/DNA spin column protocols, which can be performed on a standard laboratory benchtop. Dispose of all organic solvent waste as recommended by your local Occupational Health and Safety Administration. I recommend that even plastics and glassware that have come into contact with the aforementioned chemicals be treated as hazardous material.

Background

Drawbacks to current approaches. The extraction and subsequent purification of the major cellular macromolecular species-DNAs, RNAs, proteins, lipids, and other (polar)

metabolites- from reef-building corals (**Fig. 1**) and their dinoflagellate endosymbionts (**Fig. 2**) is still in its infancy, despite publications on these topics dating back into the 1980s (e.g., Schoenberg & Trench 1980a-b). This statement would appear to be at odds with the fact that many hundreds of peer-reviewed articles have been produced on coral gene expression alone (e.g., Mayfield et al. 2012a, 2013c, 2014a-b); has our field reached the state-of-the-art level of analysis that was once only realized by those working on model organisms, such as mice? Unfortunately, this is not yet the case. For one, over 10 years ago, Mayfield et al. (2009) revealed that the vast majority of reef coral and endosymbiotic sea anemone (which can serve as a model system for the more-difficult-to-study corals; Mayfield et al. 2014c; Chen et al. 2016) articles had either completely failed to extract macromolecules from the dinoflagellate endosymbionts (family Symbiodiniaceae) or, if they *were* extracted, they were left unanalyzed (or treated as contamination). The failure to either extract or include Symbiodiniaceae data in molecularly-focused publications of that era (e.g., Moya et al. 2008) potentially left many to conclude that these algae, which are obligate, photosynthetic mutualists without which the coral host would perish from starvation (Muscatine & Cernichiari 1969), are "background players" with respect to their contribution to the macromolecules yielded from coral tissue biopsies. Unfortunately, this "uni-compartmental" focus remains prevalent to this day (**Table 1**), and very few multi-'Omic studies have made an effort to profile macromolecular species from all members of the coral holobiont. My primary goal in this book chapter is to challenge coral biologists to more comprehensively characterize the complex molecular landscapes of their target species; sure, it may mean a more exhausting day in the laboratory, but I hope to convince you that it is worth the extra effort.

Although it is certain that the intracellular dinoflagellate populations do not contribute *more* biomass to the "holobiont" (the collective name for the association of coral, dinoflagellates, and other eukaryotic and bacterial microbes that associated with the coral colonies) than the host anthozoans in which they reside (Mayfield et al. 2019a-b), when using proper, robust extraction techniques they can actually yield as much, if not more, of the target macromolecules of interest. Perhaps this is unsurprising. After all, dinoflagellates have amongst the largest genomes ever characterized (LaJeunesse et al. 2005), meaning that, even if there were, for instance, five times more host tissue material than dinoflagellate, if the genomes are truly five times larger than those of the average coral (a good current estimate), a very similar amount of host coral and Symbiodiniaceae DNA might very well be extracted from the same biopsy; in fact, this does indeed appear to be the case (Mayfield et al. 2010).

While genome size is static within a living cell, it is more difficult to quantify or model a "typical" host coral or Symbiodiniaceae transcriptome, proteome, lipidome, or metabolome because the respective molecules are in a constant state of flux due to the metabolic needs of the cells. In the few instances in which the relative host coral/Symbiodiniaceae mRNA ratios were reported (e.g., Mayfield et al. 2011; **Table 1**), it is not uncommon for the dinoflagellates to contribute over 1/3 of all extracted RNA in a healthy reef coral. Those looking to model the health of a reef coral, then, would be wise to include the dinoflagellate contigs in their bioinformatic analyses (*sensu* Mayfield et al. 2014d), though to this date, the more common reductionistic approach is taken; researchers interested in host coral gene expression omit the Symbiodiniaceae genes while others exclusively focused on the endosymbionts omit those genes of their coral hosts (**Table 1**). Although this approach

makes sense for certain laboratory experiments, I argue that, for coral biologists seeking to predict the ultimate fates of reef-building corals (e.g., Mayfield et al. 2017a-c, 2018d, 2022), such a simplistic, uni-compartmental approach is decidedly sophomoric; instead, those interested in characterizing the health and stress tolerance of corals using molecular approaches (*sensu* Mayfield 2016) would do far better by considering all constituents of the coral holobiont, or at least the coral host and endosymbiotic dinoflagellates at a minimum.

Fig. 1. An Indo-Pacific coral reef featuring a high abundance of a branching coral species (*Acropora* sp.). Photo by the author.

The overall tendency to exclude Symbiodiniaceae molecules from 'Omics analyses, however, may not actually stem from a scientific desire to focus exclusively on one member of the association; I surmise herein that it actually follows from poor-quality extraction techniques, or, potentially more likely, the use of extraction protocols originally optimized for use with model organisms such as *E. coli*. Were the latter to be the case, one might actually expect a host coral-dominated macromolecular extract. To understand why this is the case, a more detailed understanding of coral-dinoflagellate biology is warranted. Firstly, reef-building corals and endosymbiotic sea anemones (e.g., *Exaiptasia*) house the Symbiodiniaceae dinoflagellates in only half of their cells, those of the gastroderm (Peng et al. 2011). In essence then, the coral/endosymbiotic anemone is an animal-plant chimera. The anthozoan host cells feature a cell membrane that entirely ensheaths the endosymbionts (**Fig. 2**); the endosymbionts are not swimming freely within an open space, as are the bacterial microbes in our guts, but are effectively trapped in a "symbiosome" (Mayfield et al. 2013b). From an extraction standpoint, this does not pose an issue because the anthozoan cell membranes

are decidedly flimsy and easily disrupted by manual or chemical agitation (Mayfield et al. 2012b). In fact, were one to immerse a coral polyp in freshwater, many anthozoan cells would lyse due to osmotic stress alone (Mayfield & Gates 2007), without any need of corrosive organic solvents, bead mills, or mortars and pestles

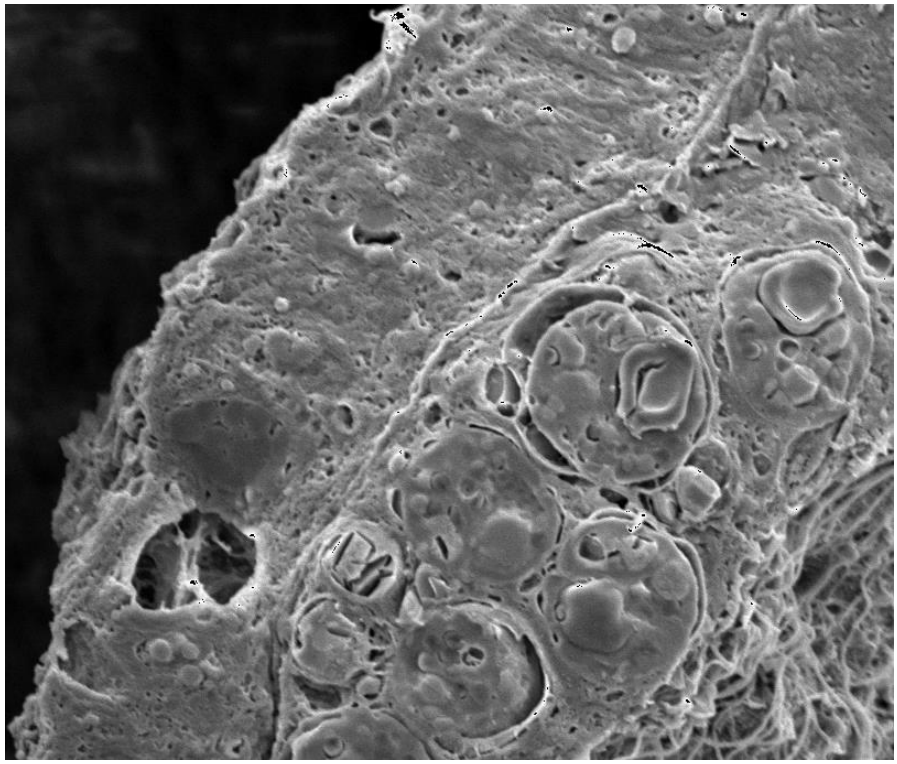


Fig. 2. A scanning electron micrograph of decalcified, freeze-fractured reef coral (*Pocillopora* sp.) tissues. The spherical objects (~10 µm in diameter) in the gastrodermal (right-most) tissue layer represent the dinoflagellate endosymbionts (family Symbiodiniaceae).

Where technique becomes more critical, though, is in the lysing of the dinoflagellate cells, which are surrounded by some of the strongest cell walls ever before studied (Lin et al. 2000). In the hypothetical example above of freshwater lysis, it is doubtful that any Symbiodiniaceae cells would lyse. This is not to say that they are resistant to osmotic stress, only that their hardy cell walls would prevent lysis. Extraction buffers featuring even guanidinium-based salts (found in virtually all commercially available spin column kits) and beta-mercaptoethanol may even be insufficient to thoroughly lyse the majority of Symbiodiniaceae cells in a coral tissue biopsy, and that is only when employed with vigorous mechanical agitation (e.g., a bead mill with acid-washed sand; Wang et al. 2013). For those researchers aiming to characterize only host coral biomolecular material, this is actually advantageous; one could realistically expect to extract *only* host coral macromolecules using these “gentler” extraction approaches. Within seconds of immersion in guanidinium-based lysis buffers (or sodium dodecyl sulfate [SDS] at 2% or greater), the vast majority of anthozoan cells will lyse, freeing the cellular macromolecules into solution to be concentrated, purified, and characterized. However, those like myself who seek to also analyze the macromolecules encumbered within the dinoflagellate cells must unfortunately use a more time-consuming, rigorous extraction approach to ensure that the incredibly resistant algal cell walls are destroyed. In other words, the extraction approach outlined in this chapter is best suited for individuals seeking to characterize RNA, DNA, proteins, lipids, and metabolites from host corals, endosymbiotic dinoflagellates, and the myriad other microbes that call the coral polyp, coenosarc, or coral colony home (**Fig. 3**). A far simpler and safer approach featuring commercially available spin columns would be better suited for researchers with an interest in host coral biological material only (not described herein).

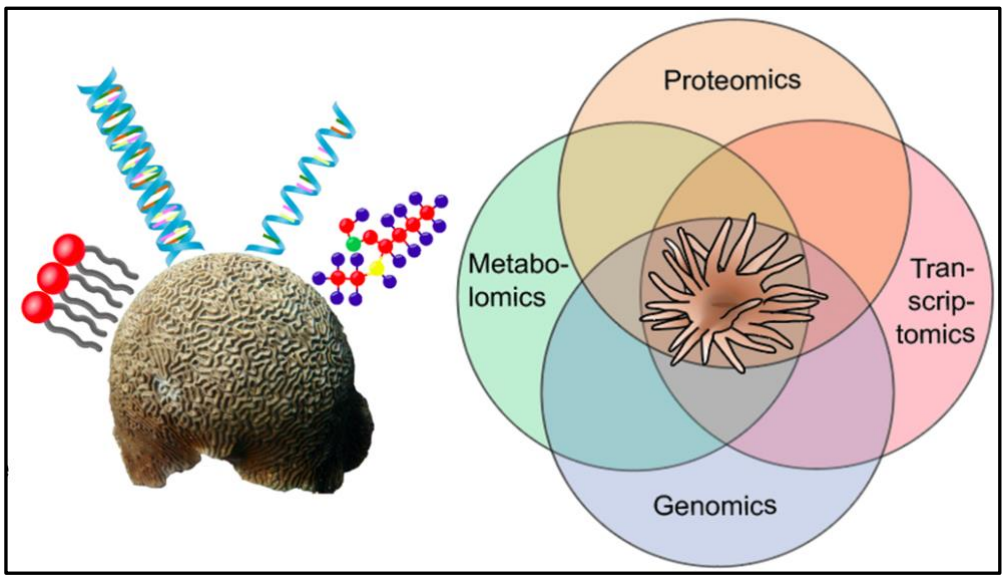


Fig. 3. A schematic featuring a brain coral (~50 cm in diameter) & a depiction of the "multi-'Omic" approach presented herein. Note that, in the Venn diagram on the right, "metabolomics" includes both polar metabolites and lipids.

Justification for a multi-'Omic approach. Although several exceptions abound (**Table 1**), another general drawback of many endosymbiotic anthozoan studies, and likely nearly all biological disciplines, is the extraction of only a singular macromolecular type from each biopsy. One might ask, "I have plentiful tissue with which to work, can I not simply extract RNA from biopsy A, DNA from biopsy B, lipids from biopsy C, etc?" For certain experiments and tissues types, this might very well be the most practical solution. Certainly, if one is interested in gene expression alone, for instance, why bother to extract the other cellular macromolecules? However, for reef-building corals, a multi-'Omic approach is recommended for at least two reasons. First, numerous reef-building corals associated with multiple dinoflagellate endosymbiont types (Mayfield et al. 2016a), not to mention a plethora of other microbes (Wegley et al. 2007). Even were one interested only in functionally profiling lipids, metabolites, mRNAs, or proteins, there is still good reason to co-extract, at minimum, the DNAs such that these complex microbial communities can be analyzed in tandem. Indeed, that was exactly what was done in the first multi-'Omic analysis of a reef coral (Mayfield et al. 2011). Secondly, although gene expression research is far more popular given growing dataset sizes and diminishing sequencing costs, there is no correlation between mRNA levels and concentrations of the proteins they encode in the few studies in which such correlation has been experimentally tested (Mayfield et al. 2016b-c, 2018a-b). As such, those looking to understand the physiological implications of climate change or other environmental changes on reef corals and their diverse microbial communities should at least profile protein concentrations and characterize the identity of the holobiont from genetic or even meta-genomic co-analysis of the DNAs. More generally, then, reef coral biologists are among the most justified in employing a multi-'Omic approach in their research projects.

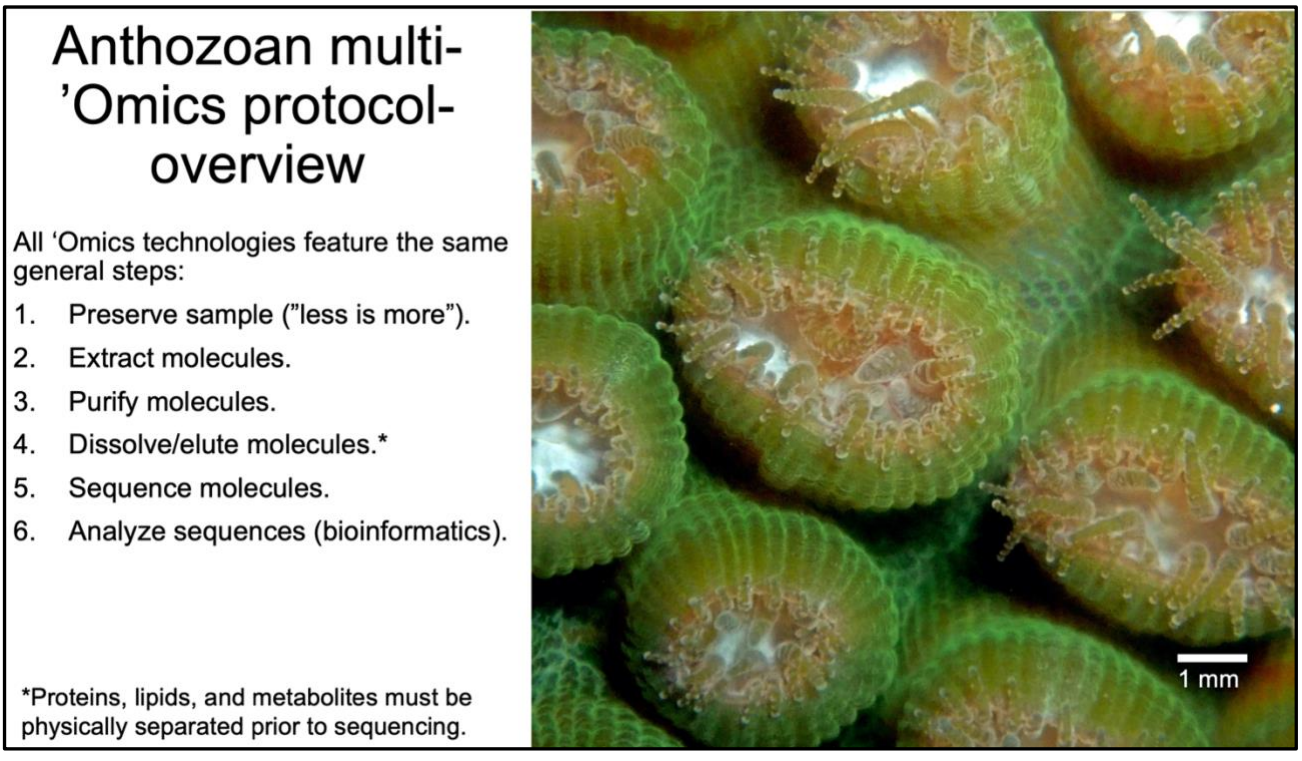
Protocol overview and rationale

Analysis of complex suites of macromolecules-namely RNAs, DNAs, proteins, lipids, and polar metabolites- of biological specimens involves the following steps (**Fig. 4**): 1) preservation of the biological specimen (i.e., biopsy), 2) extraction of target macromolecules from the cells, 3) separation of the macromolecules from one another, 4) concentration of the macromolecules, 5) washing of the macromolecules, and 6) solubilization of the

macromolecules. Although myriad options abound, I recommend and will consequently describe two over-arching approaches: Option A (**Fig. 5**) and Option B (**Fig. 6**). The former is superior in that the proteins are far higher in quantity relative to Option B. The major disadvantage of Option A is that polar metabolites are co-isolated with RNAs and DNAs and must be subsequently pooled, concentrated, and purified; this results in a large number of steps, as well as many dozens of microcentrifuge tubes to track. In contrast, these molecules are separated with a modified Bligh and Dyer extraction in Option B, which is derived from a seminal work by Podechard et al. (2018). However, using this approach the proteins form a lens between the lipids and polar metabolites, and it is difficult to re-dissolve these irreversibly denatured proteins. They may, then, only be suitable for normalization (i.e., normalizing lipid & metabolite levels to total protein) and not for proteomic analyses (I have never used the proteins from Option B for shotgun or label-based proteomics.).

With the exception of the proteins of Option B, this protocol consistently yields high-quantity and high-quality macromolecules from a diverse array of marine organisms though has only ever been *thoroughly optimized* for reef-building corals (both larvae lacking in skeletal material & adult corals featuring large quantities of calcium carbonate skeleton) and model anthozoan-dinoflagellate endosymbioses, namely *Exaiptasia* spp; differing sample types may require slight adjustments. Please note that trade-marked reagents and chemicals do not necessarily constitute a formal endorsement over generic or similar products from other vendors; these simply reflect those with which I have the most experience.

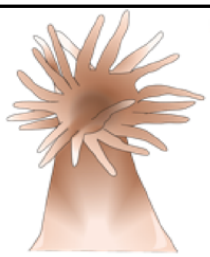
Fig. 4. An overview of the multi-'Omic extraction & representative coral sample material. For scleractinian corals with large corallites (e.g., those of the *Montastrea cavernosa* colony in this figure), a single polyp yields sufficient RNA, DNA, protein, lipid, and metabolite quantities for all manner of 'Omic analyses. Small-polyped corals (~1 mm; e.g., pocilloporids) may instead necessitate 15-30 polyps to yield sufficient protein, in particular.



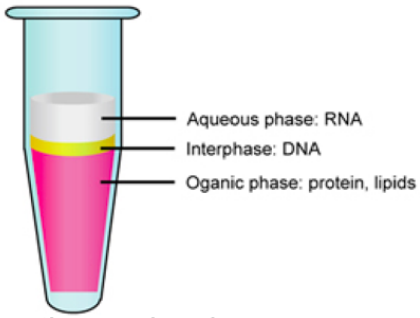
THE PROTOCOL

Option A:

RNA
DNA
Proteins
Lipids+metabolites



+TRIzol



1. Homogenize to lyse cells
2. Add chloroform to separate RNA from other molecules
3. Add back extraction buffer to separate DNA from remaining molecules
4. Add acetone to precipitate proteins and centrifuge

pellet=protein
5. Wash repeatedly
6. Dry partially
7. Dissolve

supernatant=lipids+metabolites
5a. Dry under stream of nitrogen gas
6a. Wash
7a. Dissolve

8. Separate purified molecules via chromatography (liquid or gas)
9. Profile/sequence by mass spectrometry

Pros	Cons
RNA, DNA, & protein are high quality	Lipids & polar metabolites are inter-mixed Some polar metabolites partition w/RNA

Fig. 5. Endosymbiotic anthozoan multi-'Omic extraction approach-option A. Please note the then cartoon coral polyp has been magnified approximately 10-fold relative to the adjacent cartoon of the microcentrifuge tube. Please note that it is possible that a new solvent system could be exploited to separate lipids from polar metabolites (between steps 4 & 5a); admittedly, I have not yet tested this.

SAMPLE COLLECTION

Note: Alcohols (e.g., 70% ethanol) are not effective preservatives for RNAs or proteins and should be avoided.

1. A diverse array of methods can be used to sample adult corals, though two have been consistently shown to *under*-perform the others: tissue "blasting" using compressed air or water picks and tissue "scraping" with razor blades or scalpels. One issue with the former is that the tissue removal process itself is so lengthy (several minutes) that macromolecular changes can occur. Side-by-side comparisons of tissue+skeleton extractions (from simply cutting adult corals with bone-cutting pliers) vs. extracting RNAs, DNAs, and proteins from water-picked coral tissues (Mayfield et al. 2013a) highlight that the RNAs in particular suffer with the latter approach.
   A: Although scraping tissues off of coral skeletons with sterile razor blades or scalpels could be effective if done quickly, there are two issues:
      I- the intensive handling of the fragment/biopsy (or colony) needed to take the sample elicits substantial mucus generation from the coral polyps;
      II- this mucus can then function as a barrier to the fixative/preservative in which the tissue will be stored, meaning metabolic activity may not be halted until the samples are fully frozen.
   B: Instead, cutting corals with bone-cutting pliers, drills, or other means, in which

skeleton and tissue are collected in tandem almost without exception will lead to higher yield and quality of macromolecules.

C: A good starting point in terms of amount of starting material is a single 1-cm diameter polyp or 10-20 polyps of 1 mm in diameter.

D: Larvae can simply be aliquoted into tubes containing TRIzol or flash-frozen in liquid nitrogen (LN2).

E: If there is scientific justification to avoid the tissue-skeleton interface, then the two aforementioned skeleton-free approaches will likely be the only options since:

I- removing tissues chemically (HCl or bleach) or

II- dissolving the skeleton with EDTA (as is used for histological preparation) lead to far too rampant molecular degradation to be suitable for sequencing-based analyses.

2. Freeze samples in LN2 (or charged dry shipper at -150°C) and store at -80°C (or in the dry shipper itself if the extraction will be done within several weeks of sampling). Alternatively, samples can be immersed directly in TRIzol or similar acid phenol-guanidinium solvent and stored frozen (-80°C) until the time of extraction.

A: When preserving large pieces of adult coral, such as entire branches or "plugs" (i.e., mini-cores) made from pneumatic drills, LN2 immersion is almost certainly the preservation method of choice; the use of corrosive preservatives would necessitate bringing them to the diving vessel or aquarium wet lab in large quantities (since the biopsies must be fully immersed).

B: Care should be taken to avoid prolonged periods between harvesting of the biopsies and immersion in preservative (be it a solvent or LN2); gene expression

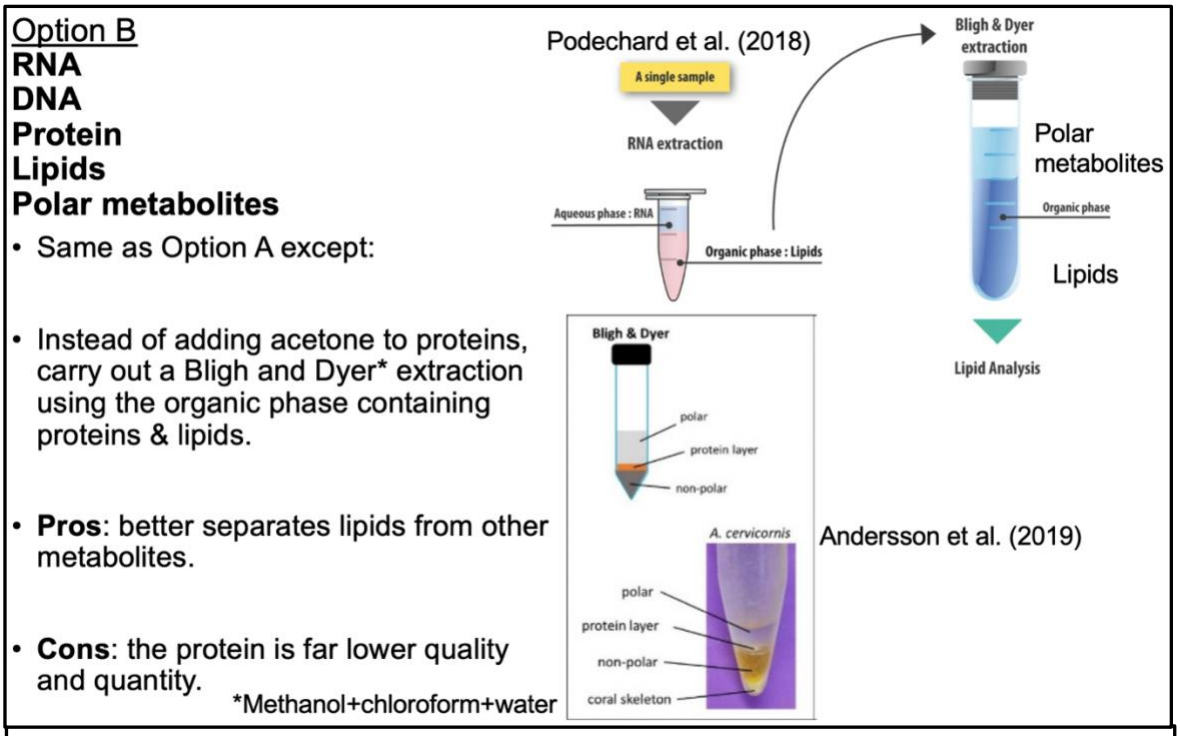


Fig. 6. Endosymbiotic anthozoan multi-'Omic extraction approach-Option B. Please note that these images have been modified from the respective publications (both of which are open-access & cited in the reference list). *A. cervicornis=Acropora cervicornis* (a reef-building coral that is the primary focus of reef restoration efforts in South Florida, USA).

and protein concentrations can change within minutes of the collection process itself (be it via hammer & chisel, pneumatic drill, bone-cutting pliers, or razor blades for adult corals, or by mere pipetting for coral larvae or small sea anemones).

C: Although superior to water or alcohols, RNALater® (TFS) is a poor preservative for adult corals in particular due to the near-immediate precipitation of the constituent salts upon contact with marine osmoconformers. A newer generation of "shield" reagents from Zymo are said to yield high-quality RNA from adult reef corals, though this has yet to be demonstrated in the peer-reviewed literature as of May 2022. Were this to be later proven, this could represent an attractive alternative since these reagents are far less hazardous than TRIzol and much less cumbersome to transport than a LN2 dry shipper or dewar.

RNA EXTRACTION

Note 1: Attempt only 8-12 extractions/day.

Note 2: See safety recommendations above.

Note 3: These steps are identical between Option A (Fig. 5) and B (Fig. 6).

1. See "Sample Collection" section above.
2. Remove samples from freezer, and, if necessary, quickly break off a small (~50 mg) piece from the frozen biopsy with sterile bone-cutting pliers.
   A: Skip this step if samples were previously frozen in TRIzol.
   B: If samples were frozen in TRIzol or equivalent, thaw sample+TRIzol and transfer into mortar (Option 1) or 2-ml bead mill tube (Option 2).
3. **Option 1**: Quickly cover coral fragment with additional TRIzol (or similar phenol-guanidinium-based solution) to 1.5 ml total volume and homogenize for several minutes with a mortar and pestle (until the solution becomes a translucent pink) OR **Option 2**: Transfer tissue sample to 2-ml tube compatible with bead mill (e.g., MP Biomedical's FastPrep™ series), quickly overlay with TRIzol to ~1.5 ml total volume, and add several large steel or numerous, small steel (or ceramic) ball bearings (depending on dimensions of biopsy).
   A: Apply upwards of five, 30- or 60-s runs of the bead mill at ~4-6 m/s.
   B: Place tubes on ice for 5 min after each run to allow samples to cool (heat builds up during bead beating.).
4. Transfer 1 ml of homogenized coral tissues in TRIzol to a new 1.5 ml microcentrifuge tube, vortex, and incubate samples @ room temperature (RT) for 5 min.
   A: Samples can be stored in TRIzol for several weeks at -20°C.
   B: Long-term storage in TRIzol at -80°C is also suitable for proteomic analyses.
   C: Residual TRIzol+tissue can be transferred to a separate tube to be used as a backup and frozen at -80°C
5. Add 200 µl of chloroform (without amylenes) to each sample and incubate @ RT for 2-10 min.
6. Centrifuge (hereafter "spin") samples @ 12,000 *xg* for 15 min @ 4°C.
7. Remove aqueous phase (~600 µl) and add to new 1.5 ml microcentrifuge tube containing 250 µl of isopropanol.
8. Add 250 µl of high salt solution (HSS; 0.8 M Na citrate+1.2 M NaCl in DEPC-treated water), vortex, and incubate @ RT for 10 min or for longer periods (several hours-months) at -20°C or colder.
   A: If you do not plan to undertake RNA-based analyses in the coming days, stop here and store samples precipitated in the -80°C freezer; otherwise, proceed with step 9.
   B: The HSS will precipitate after long-term RT storage and should be remade often.

Table 1. Simple-random-sample of 17 endosymbiotic anthozoan molecular biology articles. Unless otherwise noted, all studies were undertaken with adult scleractinian corals. All trade-marked reagents and kits are from Thermo-Fisher Scientific except NucleoSpin® (Machery-Nagel) and Aurum™ Total RNA minikit (Bio-Rad). NA=not applicable. NR=not reported.

Reference	Molecules characterized	Extraction approach	Consi-dered anthozoan host?	Considered dino-flagellates?	Host/symbiont molecular ratio	Molecular integrity reported?
Mayfield (2022)	RNA, DNA, Protein	TRIzol®+mortar & pestle	Yes	Yes	~2:1	Yes
Mayfield & Dempsey (2022)	RNA, DNA	TRIzol®+mortar & pestle	Yes	Yes	~2:1	Yes
Rubin et al. (2021)	RNA	NucleoSpin® TriPrep+mortar & pestle+bead mill	Yes	Yes	~1:2	No
McRae et al. (2021)[a]	Protein	TRIzol®+mortar & pestle	Yes	Yes	~14:1	Yes
Tisthammer et al. (2021)	Protein	Urea+tissue shearer	Yes	No	NA	No
Sproles et al. (2019)[b]	Protein, DNA	LN2+mechanical homogenization	Yes	No	NR	No
Rocker et al. (2019)	RNA, Lipid	Aurum™ Total RNA minikit, 2:1 dichloromethane:methanol	Yes	No	NA	No
Dimos et al. (2019)	RNA	RNAqueous®	Yes	No	NA	Yes
Lohr et al. (2019)	Metabolites	Methanol	Yes	No	NA	No
Wright et al. (2019)	RNA	RNAqueous®+bead mill	Yes	Yes	~9:1	No
Seveso et al. (2017)	Protein	SDS buffer+mortar & pestle[c]	Yes	No	NA	No
Chen et al. (2017)	Lipid, Protein	Bligh & Dyer	Yes	Yes	NR	No
Ricaurte et al. (2016)	Protein	Rehydration buffer+mortar & pestle	Yes	No	NA	No[d]
Vidal-Dupiol et al. (2013)	RNA	TRIzol® (details not reported)	Yes	No	NA	No
Barshis et al. (2013)	RNA	TRIzol®+bead mill	Yes	No	NA	No
Putnam et al. (2013)	RNA, DNA, Protein	TRIzol®+mortar & pestle	Yes	Yes	~2:1	Yes
Mayfield et al. (2011)	RNA, DNA, Protein	TRIzol®+mortar & pestle	Yes	Yes	~2:1	Yes
Kenkel et al. (2011)	RNA	RNAqueous®+razor blade	yes	no	NA	yes

[a]Analyzed both adult and larval corals. [b]Used endosymbiotic sea anemones. [c]Erroneously claims to be a dinoflagellate free preparation method (not demonstrated). [d]Proteins appear degraded on the gel images presented.

Table 2. Non-standard abbreviations and chemical information. Please note that this does not constitute the full list of reagents, chemicals, and materials necessary to carry out this protocol, nor are catalog numbers generally presented throughout the chapter since they are prone to changing rapidly. All reagents and chemicals should be "molecular-grade" rather than ACS or "reagent-grade." Ensure that chloroform does not contain stabilizing agents (e.g., amylenes). NA=not applicable.

Full term	Abbreviation	Manufacturer
Acetonitrile	ACN	Various (e.g., Sigma-Aldrich)
Ammonium bicarbonate	AB	Various (e.g., Sigma-Aldrich)
Back extraction buffer	BEB	See protocol below.
Bovine serum albumin	BSA	Various
Butylated hydroxytoluene	BHT	Various
Diethyl pyrocarbonate	DEPC	Various
Dithiothreitol	DTT	Various
Ethylenediaminetetraacetic acid	EDTA	Various
High salt solution	HSS	NA: make in-house
Hydrochloric acid	HCl	Various
Isobaric tags for relative & absolute (protein) quantification	iTRAQ	Sciex
Mass spectrometry/spectrometer	MS	Thermo-Fisher Scientific (TFS)
Non-fluorescent	NF	NA
Room temperature	RT (25°C)	NA
Sodium	Na	NA
Sodium chloride	NaCl	Various
Sodium dodecyl sulfate	SDS	Various
Tandem mass tags	TMT	TFS
Triethyl ammonium bicarbonate	TEAB	TFS
Trifluoroacetic acid	TFA	Various
Tris-2-carboxyethyl-phosphine	TCEP	Various
Tris-borate-EDTA	TBE	Various
Tris-EDTA	TE	Various

9. Spin @ 12,000 *xg* for 10 min @ 4°C.
10. Decant supernatant (~1 ml) containing salts, isopropanol, and some polar metabolites into 15-ml glass culture tube with Teflon-coated lid and conical bottom (e.g., Pyrex® culture tubes) capable of being spun at high speeds (e.g., in an Eppendorf 5810R centrifuge run at 1,500 *xg*) and store at -80°C.
11. Re-suspend the RNA pellet in the minimum volume of lysis buffer recommended by your preferred RNA clean-up spin column kit.
    A: This is typically 350-500 µl.
    B: Axygen, Zymo, Protech, and numerous other kits have been used successfully.
    C: You may need to use the pipet tip to completely break apart the pellet.
12. After breaking apart the pellet via pipetting or vigorous vortexing, follow the kit manufacturer's recommendations; typically first involves adding an equal volume of 100% ethanol to the RNA pellet in lysis buffer prior to loading the spin column.
13. Wash and DNase treat RNA on-column (15-20 min) as recommended by the manufacturer.
14. After decanting final wash, incubate spin column in new microcentrifuge tube in a 60°C oven for 5-10 min to evaporate residual ethanol in column.

15. Elute RNA in 30-50 µl of DEPC-treated or nuclease-free water after letting the water incubate on the column for 5 min prior to the final spin.
16. Assess RNA quantity on a mini-spectrophotometer (e.g., NanoDrop; duplicate readings of 2 µl/reading), bioanalyzer, Qubit, or other means.
    A: DNA-free RNA using this protocol is typically between 30-300 ng/µl (1-10 µg of total RNA) when using a 50-100-mg piece of coral (including skeleton).
    B: The 260/280 should be between 1.8-2.2; If it is too low, there may be protein contamination. If it is too high, the RNA may be degraded.
    C: The 260/230 should be >1; if it is far lower, alcohol contamination is likely.
17. Electrophorese ~5 µl of RNA on a 0.8% Tris-borate-EDTA (TBE)-agarose gel stained w/ SYBR® Gold or SYBR® Red (avoid highly mutagenic stains like ethidium bromide).
    A: Alternatively, a formaldehyde (denaturing) gel may be used to better estimate the size of 28 and 18s bands; mRNA should be a 0.5-12 kb smear.
    B: The best option for RNA integrity analysis is a bioanalyzer, and many sequencing cores now bundle this with their sequencing costs.
18. Dilute RNA to desired concentration (e.g., 20 ng/µl) with DEPC-treated water and store at -80°C.

DNA extraction

Note 1. This protocol can be performed simultaneously with the final steps of the RNA extraction.

Note 2. This protocol is identical between Option A (Fig. 5) and B (Fig. 6).

1. Remove remaining RNA aqueous phase (see step 7 of RNA extraction.).
2. Add 500 µl of back extraction buffer ("BEB;" 1 M Tris base, 4 M guanidinium thiocyanate [*not* guanidine HCl], & 50 mM Na citrate).
3. Vortex and place tubes on shaker table or, preferably, a tube mixer (e.g., ELMI's RM2 Intelli-mixer) for 10-20 min.
   A: DNA can be left on shaker table/Intelli-mixer for 1-2 hr, but no longer.
   B: Samples should *not* be frozen at this stage.
4. Spin samples @ 12,000 *xg* for 15 min @ 4°C.
5. Transfer aqueous phase (~600 µl) to a new microcentrifuge tube.
6. Precipitate DNA w/ 2 µl Pellet Paint-NF™ (Millepore; only necessary for tissue samples <50 mg), 60 µl of Na acetate (3 M, pH 5.2), and 600 µl (1 vol) of isopropanol @ RT for 10 min or @ -20°C for longer periods (up to several months).
   A: Isopropanol alone can be used instead.
   B: If you have no immediate need for the DNA, *stop here* and precipitate at -20°C or -80°C. Otherwise, proceed with step 7.
7. Spin @ 12,000 *xg* for 10 min @ 4°C.
8. Decant supernatant with salts, isopropanol, and some polar metabolites (~1 ml) into the 15-ml glass tube containing polar metabolites in isopropanol from step 10 of the RNA extraction.
9. Resuspend DNA pellet in minimum recommended lysis buffer volume from preferred DNA cleanup spin column kit (normally at least 100 µl).
   A: PCR and DNA clean-up kits from Qiagen, Zymo, Axygen, Protech, and other vendors have been used successfully.
   B: Use pipet tip to break apart DNA pellet and vortex until it has completely disintegrated.
10. Load spin column with entire volume of lysis buffer+DNA and spin as recommended by the manufacturer.

11. Carry out remaining spins and washes as recommended by the manufacturer.
12. Put the "eluent" (normally Tris or Tris-EDTA [TE]) in a 60°C oven for 5-10 min before adding it to the spin columns (this will help the DNA to dissolve.).
13. After the second wash, put the empty columns/tubes into the centrifuge and spin at 12,000 *xg* for 3 min to remove excess ethanol.
14. Transfer spin columns to new 1.5-ml microcentrifuge and place in 60°C oven for 5 min. This will further help to remove residual ethanol.
15. Add desired volume (~30-50 µl) of pre-warmed eluent to each column and wait for 5 min.
    A: DNA will dissolve poorly in unbuffered water using this protocol.
    B: Ensure that the eluent is either Tris (pH 8.5) or, preferably TE.
16. Perform final spin for 1 min @ 12,000 *xg* and discard spin column.
17. Quantify 2 µl of DNA x 2 times on a spectrophotometer (e.g., Nanodrop) to quantify.
    A: The DNA quantity from a 50-mg biopsy of adult coral+skeleton should be in the range of 3-30 µg (mean=~10 µg or 50 µl of 200 ng/µl).
    B: The 260/280 ratio with this protocol should be 1.7-2.
    C: The 260/230 ratio with this protocol should be >1, preferably much higher.
18. Electrophorese 5 µl on an 0.7-0.8% TBE-agarose gel (with 1X SYBR Gold or SYBR Red) at 100 V for 30-45 min.
    A: The DNA may be of such high molecular weight that it will barely move from the well.
    B: A long smear may also be seen if the DNA has degraded somewhat.
19. Dilute DNA to desired concentration (e.g.,1-10 ng/µl).
20. DNA can be stored at 4°C in TE, though storage @ -20°C is also possible if freeze thaws are kept to a minimum.

Option A-phase I: proteins (Fig. 5)

Note 1. Purification of lipids and polar metabolites using this protocol can be found in the next section ("phase II").
Note 2. If desiring lipids and polar metabolites that are better (& more easily) separated from one another (albeit with lower quality proteins), see Option B (Fig. 6) below.
Note 3. Remember that some polar metabolites have already been set aside in a 15-ml glass culture tube in the freezer (see respective steps in previous protocols).

1. After removing the DNA aqueous phase (step 5 of DNA extraction), transfer remaining ~500 µl of protein+lipids+metabolites in phenol to a new 2-ml microcentrifuge tube containing 1.5 ml acetone.
2. Precipitate proteins @ 4°C or -20°C while you finish the RNA and DNA extractions.
   A: Proteins can stay in acetone @ -20°C for several months or -80°C for several years.
   B: At minimum, precipitate @ RT for 10 min.
   C: If not immediately conducting proteomics, *stop here* and leave precipitated proteins in acetone at -80°C. Otherwise, proceed with step 3.
3. Spin samples @ 12,000 *xg* for 10 min @ 4°C and decant acetone supernatant (~ 2 ml; which contains most of the lipids & some polar metabolites) into the 15-ml glass tube containing co-extracted polar metabolites from the RNA and DNA precipitation steps described above (as reiterated in Note 3).
   A: A large protein pellet should be visible in the original 2-ml tube.
   B: Store lipids+remaining polar metabolites in acetone (~4 ml in total) at -80°C for later purification (described in more detail below under "phase II").

4. Add 1 ml protein wash I ("PWI;" 0.3 M guanidine HCl in 95% ethanol with 2.5% glycerol) and dislodge the pellet from the bottom of the tube with a pipet tip.
5. Sonicate for 10-30 min on ice until pellets are completely broken.
   A: Longer periods may be needed (~1-2 hr); properly disrupted proteins will create a "snow-like" appearance in the tubes. This is the most important step of the protocol since improperly disrupted pellets will never be effectively washed nor solubilized.
   B: Ensure that water temperature remains cool in the sonicator bath by periodically adding ice.
6. Spin samples @ 8,000 *xg* for 5 min @ 4°C and decant supernatant.
7. Perform two additional washes with 1 ml of PWI.
   A: Sonicate on ice until protein pellets dissociate completely.
   B: Subsequent sonication steps should require less time to fully break apart pellets.
   C: Proteins in PWI can be stored @ -20°C for several months.
8. After decanting the supernatant from the 3rd wash, add 1 ml of protein wash II ("PWII;" 95% ethanol w/ 2.5% glycerol) and sonicate on ice until pellets are broken.
   A: Optional step: upon full disruption of the pellet, transfer 500 µl of proteins in PWII to a new 2-ml microcentrifuge tube.
   B: Freeze at -80°C to serve as a backup.
9. Spin @ 8,000 *xg* for 5 min @ 4°C and decant supernatant.
10. Dry proteins inverted on benchtop for 5-15 min; do not over-dry pellets, as this will make them harder to dissolve.
11. The optimal solubilization buffer will depend on the application:
   A: For western blots, add ~150 µl of 1x "Laemmli" buffer (also called "sample buffer;" 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, & 0.0625 M Tris-HCl [pH 6.8]).
   B: For shotgun proteomics (described in detail below), dissolve proteins in 100-200 µl of ammonium bicarbonate (AB; 50 mM).
   C: For iTRAQ or TMT (i.e., label-based proteomics; both described below), dissolve protein in 0.5 M triethyl ammonium bicarbonate (TEAB).
   D: For 2-D gels (*sensu* Mayfield et al. 2016c), dissolve protein pellets in rehydration buffer (with thiourea); please note that, in most cases, a "next generation" proteomic approach (i.e., TMT or iTRAQ) will yield superior data for lower effort.
12. Sonicate on ice for 5-10 min.
13. Spin @ 12,000 *xg* for 5-15 min @ 4°C. If performing western blots, boil @ 100°C for 5 min before this spin.
14. If there is still a large protein pellet after this spin, that signifies that proteins did not dissolve properly.
   A: If so, add SDS (2-10% solution in water) to a final concentration of 0.067% (e.g., 6.7 µl to 200 µl of protein in 0.5 M TEAB or 0.5 M AB).
   B: Vortex vigorously on Vortex Genie or via passing the pellet through the pipet tip, and re-perform step 13.
15. Transfer supernatant in equal volumes to four different 1.5 ml microcentrifuge tubes (to reduce freeze-thawing).
   A: To a fifth 1.5-ml tube, add a ~30-µl aliquot for quantification and quality analysis.
   B: This sample can be left on ice if quantifying on the same day.
   C: Freeze remaining tubes at -80°C.
16. Quantify 20-25 µl of protein.
   A: If performing western blots or 2D gels, use the 2D-Quant™ kit (Amersham Biosciences) because this kit can quantify proteins in buffers containing strong denaturants and detergents (e.g., urea). A Qubit kit can also be used.

B: If performing proteomics, BCA or Bradford assays can be used provided that the sample is diluted ~10-fold beforehand since TEAB in particular can interfere with the absorbances at higher concentrations.

C: When starting with 50 mg of coral tissue+skeleton and eluting in 150 µl of any of the aforementioned buffers, this protocol yields protein concentrations of ~0.5-3 µg/µl.

16. Mix a 1-2-µl aliquot of protein with 2X Laemmli sample buffer (see above.) and boil at 95% for 5 min
17. Load into a PhastGel® gradient 4-15 polyacrylamide gel (GE Healthcare).
    A: Please note that this system has been discontinued.
    B: Until a similar 3D-printed gel system amenable with small protein quantities is developed, it is unclear how protein quality can be assessed prior to proteomics without sacrificing the vast majority of the extracted protein (as would be required for standard SDS-PAGE).
18. Load the gel into the Phast System (GE Healthcare) after inserting two PhastGel SDS buffer strips.
19. Run proteins (1-3 µl in 1X [final] Laemmli sample buffer) alongside 1 µl of 1 µg/µl BSA standard and 1 µl of Plus2® Pre-stained protein standard (TFS) or other such pre-stained protein standard under separation method 3.
20. After ~2 hr, wash the gel three times with water on shaker table.
21. Stain with 10-20 ml SimplyBlue™ Safe Stain (Invitrogen) for 1 hr at RT; more common Coomassie stains can be used though require first fixing the gels.
22. Pour off stain (which can be reused).
    A: Wash gel repeatedly with water until bands can be seen with the naked eye.
    B: For best results, destain overnight.
23. Take a picture of the gel against a white backdrop or on an illuminated light tray.
    A: You are hoping to see an array of bands of many different molecular weights (*sensu* **Fig. 7**).
    B: What you do *not* want to see is a low molecular weight blob at the bottom of the gel (signifying degraded proteins).

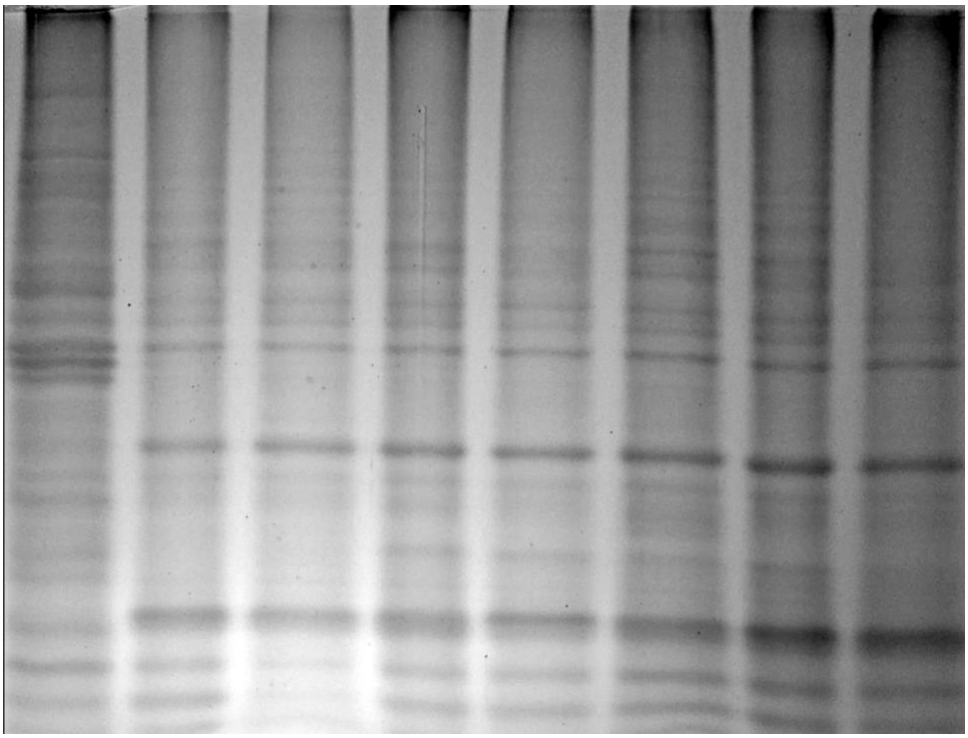


Fig. 7. Proteins (~100 µg) from the reef-building coral *Seriatopora hystrix* and its dinoflagellate endosymbionts on a 4-15% gradient SDS-PAGE gel stained with SYPRO® Ruby. Although a molecular weight ladder is not shown, the proteins range in size from ~5 kDa (bottom of gel) to 300 Kda (top). Degraded proteins are not evident.

Option A-phase II: lipids and polar metabolites (Fig. 5)

Note 1: This protocol has not been vetted to the same extent as those described elsewhere in this chapter; further optimization may be required.

1. Remove ~4 ml of lipids+polar metabolites in acetone+phenol+isopropanol+water from prior steps from the -80°C freezer and vortex.
2. Add 4 ml of methanol, 4 ml of chloroform, 3 ml of ddH_20, and 30 µl of Avanti SPLASH® LIPIDOMIX® mass spectrometry lipid standards; the latter aids in quantification and helps correct for differential lipid extraction efficiency across samples.
   A: If funding permits, I recommend spiking in non-lipid metabolite standards, as well.
   B: IROA Technologies is currently at the forefront of this initiative, though I have not yet used their metabolite standards myself.
3. Vortex vigorously and spin at 1,500 xg for 2 min.
4. Transfer bottom, organic phase containing lipids with a glass pipet to a new 10-ml glass tube.
   A: If lipids will not be analyzed immediately, add butylated hydroxytoluene (BHT) to a final concentration of 10 µM, flush with argon gas, and store at -80°C.
   B: If there is substantial insoluble material evident in the original tube upon removing the lipids, transfer the polar metabolite aqueous phase to a new, suitably-sized glass tube.
5. If lipids and polar metabolites will be analyzed on the same day, dry both to completion via inert gas evaporation (argon or nitrogen) in a fume hood or through using a speed-vac (the latter may require several hours for the ~7-7.5 ml of lipids & ~7-7.5 ml of polar metabolites to fully evaporate).
   A: Note that, because acetone and isopropanol both dissolve in water and chloroform, the exact volumes of each fraction may vary.
   B: Long-term storage of metabolites is uncommon; empirical testing may be required to determine whether storage in liquid form at -80°C (methanol will not freeze at this temperature.) is superior to freeze-drying or storing dried metabolites at -80°C.
6. Give tubes with dried lipids and metabolites to mass spectrometry core facility.
7. Normalize resulting concentrations of lipid and metabolite species to internal standards and then to total protein (calculated from the protein extraction protocol presented above); the latter is critical since it is not possible to standardize loading of biological material in an adult coral extraction.

Option B-RNA, DNA, protein, lipids, & polar metabolites

Note 1: For those interested only in proteins, lipids, and polar metabolites (& not RNA or DNA), simpler approaches could be employed (e.g., Chen et al. 2012). However, ensure that a robust enough homogenization approach is used to lyse the Symbiodiniaceae cells; simple immersion in chloroform+methanol+water is insufficient.

1. After removing the DNA aqueous phase (step 5 of DNA extraction), transfer ~0.5 ml of the organic phase containing proteins, lipids, and polar metabolites to a 10-ml glass tube with a Teflon-coated lid and conical bottom capable of being centrifuged at high speeds (e.g., Pyrex) and mix with 2.5 ml of methanol.
2. Add ~1 ml of polar metabolites in isopropanol from step 10 of the RNA extraction.
3. Add 2.5 ml of chloroform, 2 ml of water, and 10 µl of Avanti SPLASH LIPIDOMIX polar lipid standard cocktail to serve as an 1) internal standard and 2) extraction efficiency

control.
A: A metabolite standard cocktail (e.g., IROA's family of metabolite standards) should also be used as these will result in a more truly quantitative dataset.
B: Normalization to metabolite standards is an emerging field and so details have generally been omitted herein.

4. Vortex and spin at 1,500 xg for 2 min.
5. Remove the lower organic phase featuring lipids in chloroform with a glass pipet and transfer the entire volume (~3.5-4 ml) to a new glass tube.
6. Add BHT to 10 µM to limit oxidation if samples will not be analyzed the same day.
7. Regardless of date of mass spectrometric analysis, dry entire lipid volume to complete using inert gas or a speed-vac and remove all air via argon gas flushing.
8. For the polar metabolites in methanol remaining in the original 10-ml glass tube, transfer entire amount via pipet to a new 5-10 ml glass tube and either dry as for the lipids or store at -80°C (methanol will not freeze at this temperature.).
9. Add 2 ml of acetone to the remaining protein "plug" (see **Fig. 6**.) in the original 10-ml tube, vortex vigorously, and transfer entire volume of protein+acetone to new 2-ml microcentrifuge tube.
10. Precipitate protein and wash as described above in the protein protocol.
    A: As mentioned above, this protein is of lower quality than that derived from Option A; it is likely suitable only for quantification rather than for proteomics.
    B: Normalize lipid and metabolite species concentrations to the total protein of the respective sample to control for differing amounts of biomass among samples (a veritable surety for reef coral samples).
11. Consult the mass spectrometry core facility you will use for optimal solubilization buffer and volume for both lipids and metabolites, though most will gladly accept dried samples provided there is at least a rough estimate of quantity.
    A: Unlike for nucleic acids and proteins, in which quantification prior to sequencing is an integral part of the workflow (see above.), total lipid and metabolite levels are not generally known until after mass spectrometry (& even then the total quantity loaded may be hard to discern).
    B: For representative data, please see **Fig. 8**.

Shotgun proteomics

Note 1: This protocol was first published by Mayfield et al. (2021).
Note 2: Unlike for the label-based approaches discussed below, shotgun proteomics yields only presence-absence data.

1. After quantifying proteins and ensuring that they were not degraded on the mini-SDS-PAGE gels (i.e., PhastGels), take out desired quantity from the freezer.
    A: 10-100 µg will suffice, though if pH fractionation will be carried out (not discussed herein but recommended to those with larger budgets and with an increased need for maximum peptide characterization), a larger quantity may be necessary.
    B: It is not uncommon to analyze 100% of the proteins extracted (i.e., no backup material will remain).
2. Dry proteins down to 8 µl and add 15 µl of 10 M urea in 50 mM AB to denature the proteins.
3. To reduce the proteins, add 2 µl of 125 mM dithiothreitol (DTT) in 50 mM AB to the sample and incubate them at RT for 1 hr.

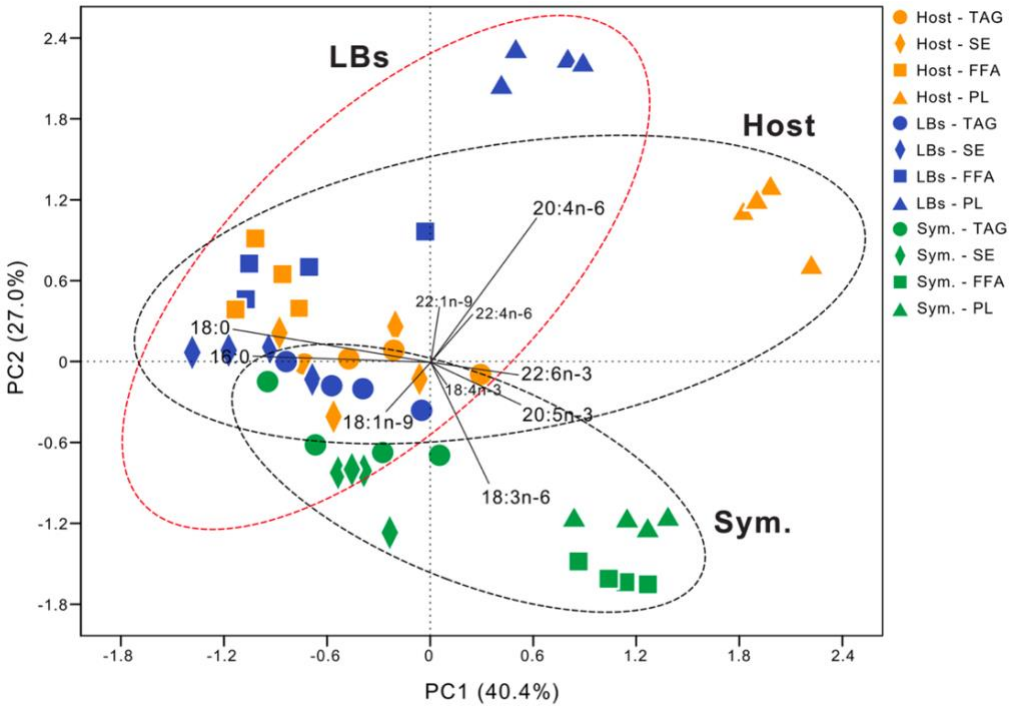


Fig. 8. A principal components analysis biplot depicting lipidomic data from an Indo-Pacific reef coral (from Chen et al. 2015). Lipids were extracted using the protocol described above from three compartments of the reef coral holobiont that were separated (pre-extraction) via centrifugation: the coral host ("Host"), the Symbiodiniaceae dinoflagellates ("Sym."), and the lipid bodies (LBs) that serve as lipid trafficking intermediaries within the holobiont. Certain fatty acid (FA) species are depicted as biplot rays, though in general the lipids were grouped into one of the four primary types: triacylglycerols (TAG; i.e., triacylglycerides), sterol esters (SE), free FA acids (FFA), and phospholipids (PL).

4. Alkylate the proteins by adding 5 µl of freshly prepared iodoacetamide (90 mM) in 50 mM AB and incubate in the dark for 30 min.
5. Quench the alkylation reaction in the dark by adding 3.33 µl of 125 mM DTT in 50 mM AB (1 hr at RT).
6. Dilute the urea to 1 M by adding 116.7 µl of 50 mM AB (total volume=150 µl).
7. Digest peptides by adding 5 µl of 0.1 µg/µl sequencing grade modified trypsin (e.g., Promega; cat. V5111) for 18 hr at 37ºC (1:30 [w:w] enzyme:protein).
8. Stop the digestion reaction by adding 7.75 µl of 50% formic acid.
9. Add 40.6 µl of 2.5% trifluoroacetic acid (TFA) to the samples (final concentration=0.5%) and check to ensure pH is <4.
10. Place a C18 spin tip (Pierce) in a 1.5 ml microcentrifuge tube and wet by adding 20 µl of 0.1% TFA in 80% acetonitrile.
    A: If using large quantities of protein (>50 µg) or if insoluble material is evident, instead use Pierce graphite spin columns.
    B: Otherwise, C18 tips will clog, making it difficult to recover proteins.
11. Spin tubes+tips at 1,000 *xg* for 1 min and discard the flow-through.
12. Equilibrate tips with 20 µl of 0.1% TFA and spin at 1,000 *xg* for 1 min.
13. Discard flow-through, add 50 µl of protein sample to the spin tip, and spin as above.
14. Repeat this step until all proteins have been passed through the spin tip.
15. Wash the protein-laden tip twice with 20 µl of 0.1% TFA and transfer to a new tube.
16. Elute proteins twice in 20 µl of 0.1% TFA in 80% ACN.
17. Speed-vac proteins to near-complete dryness.
18. Resuspend proteins in 20 µl of 2% ACN with 0.1% formic acid prior to nano-liquid

chromatography (LC), which is typically performed by a dedicated technician at a core facility; however, pertinent details can be found in the next section.

Liquid chromatography/mass spectrometry

As an example of how a shotgun proteomic approach could be taken using proteins extracted and prepared as in the previous section, I have paraphrased from Mayfield et al. (2021) as follows. First, proteins in 2% CAN with 0.1% formic acid are separated by passage through an Acclaim™ PepMap™ RSLC (75 µm × 15 cm) nanoViper column (TFS) using a Nanospray Flex ion source (TFS) on an Easy Nano LC™ 1,000 (TFS). Peptide eluates from a 2-98% acetonitrile gradient (84 min) are next run on a Q Exactive™ Orbitrap mass spectrometer (TFS) as in Musada et al. (2020). The Q Exactive (fourier transform) MS is operated in MS2 mode with "higher-energy collisional dissociation" (HCD) activation (28 eV), and the mass range scan is commonly 150-1,600 m/z. Details of the data analysis pipeline and bioinformatics can be found in Mayfield et al. (2021) for shotgun proteomics and Mayfield (2022) for iTRAQ (discussed in the next section).

iTRAQ

Isobaric tags for relative and absolute (protein) quantification (iTRAQ) is a label-based approach in which digested peptides are given an isobaric tag (i.e., label) that is detected by the mass spectrometer (MS) and used as a proxy for concentration (analogous to the more popular RNA-Seq). In the first pass of the MS, the mass peaks of the peptides are obtained. Then, in the second pass, the iTRAQ labels are analyzed (hence why you sometimes see "MS/MS" in the literature); eight samples can be analyzed in parallel. Below I have included a protocol that, while satisfactory in yielding quantitative data for several hundred-thousand proteins in a single sample, is nevertheless characterized by poor labeling efficiency (5-20% of peptides receive an iTRAQ label.); whether this is an issue inherent with the labels themselves or the protocol cannot yet be known. This approach has been used to analyze reef coral protein profiles since at least 2020 (Mayfield 2020; Reynolds et al. 2020); please see **Fig. 9** for representative data.

1. Dilute all proteins in 0.5 M TEAB to the same concentration in a 30-µl volume.
   A: A good target amount is 50-150 µg in 30 µl of 0.5 M TEAB (i.e., 1.5-5 µg/µl).
   B: AB is incompatible with this protocol because of how the labeling step is actualized.
   C: A speed vac will almost surely need to be used to achieve these high concentrations.
2. Add SDS to a final concentration of 0.067% (e.g., 1 µl of 2% SDS to 30 µl of protein in TEAB).
3. Add 1 µl of tris-2-carboxyethyl-phosphine (TCEP) to reduce the dissolved proteins' disulfide bonds.
4. Vortex and spin samples at 15,000 RPM for 5 min (hereafter referred to as "spun").
5. Incubate at 60°C for 1 hr and spin samples again.
6. Alkylate samples with 1 µl of freshly prepared 84 mM iodoacetamide in water or 0.5 M TEAB.
7. Vortex, spin, and incubate in the dark (in aluminum foil) at RT for 30 min.
8. Spin samples again and mix with 10 µl of 0.1 µg/µl sequencing grade modified trypsin (e.g., Promega cat. V5111) for 3 hr at 37°C.
9. Add an additional 1 µl of trypsin and complete digestion overnight at 37°C.
10. After spinning, dry samples (~43 µl) to 30 µl in the speed vac.

11. Resuspend dried proteins in 0.5 M TEAB (without SDS).
12. Mix proteins with 50 µl of isopropanol and 17-22 µl of the appropriate iTRAQ reagent (Sciex iTRAQ Reagent 8-plex 25 U kit) according to the manufacturer's recommendations (exact volumes vary by lot).
13. Vortex samples, spin, and incubate at room temperature for 2 hr.
14. Quench reactions with 100 µl of water for 30 min and dry to 10-20 µl in the speed vac.
15. Combine all eight samples of same batch into one tube and dry to completion.
16. Resuspend in 100 µl of water, vortex, spin, and dry to completion.
17. Repeat step 17 two more times.
18. After the last wash, dry to 30 µl in the speed vac and mix with 30 µl of 2.5% TFA.
19. Purify acidified proteins with Pierce graphite columns (for samples with <100 µg protein in total) according to the manufacturer's protocol to remove residual buffers, salts, and/or insoluble material.
20. Resuspend purified and labeled peptides in 2% ACN with 0.1% formic acid prior to nano-LC on an Easy Nano LC 1000™ TFS.
    A: The resuspension volume will normally be established by the MS core, but is likely in the range of 10-50 µl.
    B: Subsequent nano-LC/MS analyses are normally performed by a core MS facility (see abbreviated protocol above.).
21. Run peptide eluates from a 2–98% ACN gradient individually on a Q Exactive Orbitrap LTQ MS (TFS).
    A: This protocol has not been attempted using non-Orbitrap instruments.
    B: Please see Mayfield (2020, 2021, & 2022) for a detailed treatise on the bioinformatics associated with the raw MS data generated by the Orbitrap.

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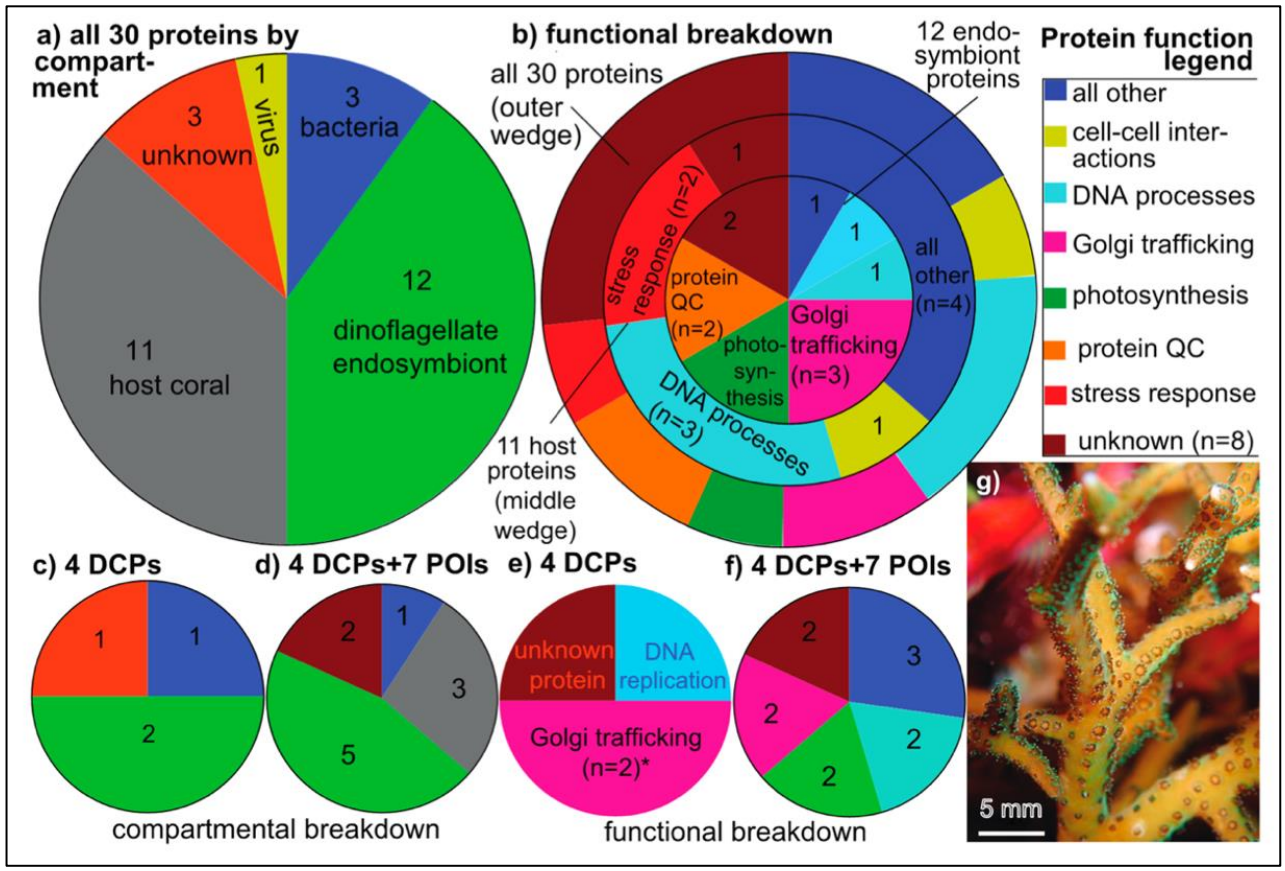
Fig. 9. Representative proteomic data obtained using the protein extraction and iTRAQ protocols outlined in this chapter (adapted from Mayfield 2020). Please note that the 30 proteins (11, 12, & 7 from the host coral, Symbiodiniaceae dinoflagellates, & other microbial constituents of the coral holobiont, respectively) represent a highly reduced subset of the initial number obtained from the mass spectrometer since several, highly stringent quality control (QC) steps were enacted; for details on the associated bioinformatics and post-peptide identification QC, please consult Mayfield (2020). DCP=differentially concentrated protein. POI=protein of interest (a term used to distinguish proteins that were useful in predictive model building but not necessarily differentially concentrated across treatments).



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Appendix

Appendix sheet 1. Printable protocols for RNA, DNA, and protein extraction. Note that the blank spaces are to be used to either place check marks (to demonstrate completion of the respective step) or to fill in pertinent details, namely with respect to times and volumes. Alternative RNA and DNA spin column kits can be substituted.

RNA EXTRACTION **Date:** _____

HOMOGENIZATION AND PHASE SEPARATION

1. Homogenized in: liquid N_2_____ TRIzol®_____ TRI-Reagent®_____ Other_____

    a. w/: mortar and pestle___ micro-pestle___ tissue lyser__ bead mill__ other___
2. Incubated @ _____ for _____ after vigorous vortexing.
    a. w/: shaker table_____ tissue lyser_____
3. Added 200 µl of chloroform and incubated @ RT for _____. w/: new tube_____
4. Spun @ 12,000 x g for 15 min @ 4°C and transferred aqueous phase to new tube.

PRECIPITATION

5. Precipitated w/ 250 µl of isopropanol and 250 µl of high salt solution @ ____ for ___.

   and spun @ 12,000 x g for 10 min @ 4°C. a. w/: Pellet Paint™ _____

PURIFICATION

6. Re-suspended pellet in 350 µl of Planet Lysis buffer A and 350 µl of 100% ethanol.
7. Added 700 µl to GeneMark Plant Total RNA kit spin column.
8. Followed manufacturer's recommendations and incubated columns in 60°C oven.
    a. On-column DNase digestion_____
9. Eluted into _____ µl of DEPC-treated water.

DNA EXTRACTION

PHASE SEPARATION

1. Added 500 µl of back extraction buffer & incubated on shaker table for _____.
2. Spun @ 12,000 x g for 10 min @ 4°C & transferred aqueous phase to new tube.

PRECIPITATION

3. Precipitated DNA w/ 60 µl of 3 M NA acetate & 600 µl of isopropanol @ ____ for ___.
    a. w/: 2 µl of Pellet Paint™_____(aids in visualizing pellet for low-yield samples).
4. Spun @ 12,000 x g for 10 min @ 4°C.

PURIFICATION

5. Re-suspended pellet in a minimum volume of 100 µl of the first buffer of the preferred DNA clean-up kit (e.g., PCR-A buffer from Axygen's PCR clean-up kit).
6. Carried out spins and washes as recommended by manufacturer.
7. After evaporating residual ethanol in 60°C oven, eluted DNA into ___µl of pre-warmed (to 60°C) "eluent" (must include Tris at a pH of at least 8 for best DNA elution).

PROTEIN EXTRACTION

8. Precipitated protein in 1.5 ml of acetone @ _____ for _____.
9. Spun @ 12,000 x g for 10 min @ 4°C.
10. Washed pellet 3x w/ PWI and 1x w/ PWII @ 8,000 x g for 5 min @ 4°C, and dried on benchtop for _____ min.
11. Re-suspended protein in _____µl of_____ buffer, sonicated for _____ min, boiled for ____ min @ 100°C, and transferred _____ul to each of ____tubes.
12. Quantified ____µl with the 2-D Quant kit, ____with a Bradford assay, ____with a BCA assay, or ____with the Qubit protein assay kit.

Appendix sheet 2. Printable sheets for RNA, DNA, and protein quantification and quality control analyses. A spectrophotometer or, preferably, a bioanalyzer can be used to generate these, or comparable, data. Subscripts correspond to the technical replicate number. BSA=bovine serum albumin (the most common standard used in protein quantification assays).

RNA									
Sample name	**$[RNA]_1$**	**$260/280_1$**	**$260/230_1$**	**$[RNA]_2$**	**$260/280_2$**	**$260/230_2$**	**$[RNA]_{avg}$**	**$260/280_{avg}$**	**$260/230_{avg}$**
DNA									
Sample name	**$[DNA]_1$**	**$260/280_1$**	**$260/230_1$**	**$[DNA]_2$**	**$260/280_2$**	**$260/230_2$**	**$[DNA]_{avg}$**	**$260/280_{avg}$**	**$260/230_{avg}$**

PROTEIN

Sample	Absorbance @ ___ nM (depends on assay)	ng/assay volume	[protein] ng/µl	Total protein	Total protein-post QC
0 BSA					
10 BSA					
20 BSA					
30 BSA					

40 BSA					
50 BSA					
Average					