



Porphyrin Detection in Denatured Cnidarian Tissue Extracts



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About the CDHC

The Coral Disease and Health Consortium (CDHC) is a Working Group of the U.S. Coral Reef Task Force charged with organizing and coordinating the scientific resources of the U.S. and its territories to meet the challenge presented by globally declining coral reefs. Its mission is to preserve and protect the health of coral reef ecosystems through an understanding of the effects of natural and anthropogenic stressors on reef-building communities. The CDHC serves to unify the coral health and disease research community, identify research priorities, and encourage a new generation of coral researchers through education and outreach. The biomedical perspective and innovative technologies developed from Consortium efforts is envisioned to give scientists, resource managers, and industry new tools to identify and alleviate hidden stresses before they become environmental health crises. Currently over *125 partners*, including federal agencies, NOAA, DOI, EPA, along with academia, non-profit and industry, contribute their time and expertise to the CDHC, while organizational infrastructure is supported by the congressionally funded NOAA Coral Reef Conservation Program.

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Porphyrin Detection in Denatured Cnidarian Tissue Extracts

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Overview

Porphyrin metabolic disruption from exposure to xenobiotic contaminants such as heavy metals, dioxins, and aromatic hydrocarbons can elicit overproduction of porphyrins. Measurement of porphyrin levels, when used in conjunction with other diagnostic assays, can help elucidate an organism's physiological condition and provide evidence for exposure to certain toxicants. A sensitive microplate fluorometric assay has been optimized for detecting total porphyrin levels in detergent solubilized protein extracts from symbiotic, dinoflagellate-containing cnidarian tissues. The denaturing buffer used in this modified assay contains a number of potentially interfering components (e.g., sodium dodecyl sulfate (SDS), dithiothreitol (DTT), protease inhibitors, and chlorophyll from the symbiotic zooxanthellae), which required examination and validation. Examination of buffer components were validated for use in this porphyrin assay; while the use of a specific spectrofluorometric filter (excitation 400 ± 15 nm; emission 600 ± 20 nm) minimized chlorophyll interference. The detection limit for this assay is 10 fmol of total porphyrin per μg of total soluble protein and linearity is maintained up to 5000 fmol. The ability to measure total porphyrins in a SDS protein extract now allows a single extract to be used in multiple assays. This is an advantage over classical methods, particularly when tissue samples are limiting, as is often the case with coral due to availability and collection permit restrictions.

Introduction

The word cnidarian (Phylum Cnidaria) comes from the Greek word *cnidos*, meaning stinging nettle. All four classes of cnidarians (Anthozoa, Scyphozoa, Cubozoa, Hydrozoa) possess characteristic stinging cells or cnidoblasts. In this document, anthozoans are represented by anemones and stony corals in Subclass Hexacorallia (e.g., *Acropora palmata*) and soft corals in Subclass Octocorallia (e.g., *Leptogorgia virgulata*) (Fig. 1).

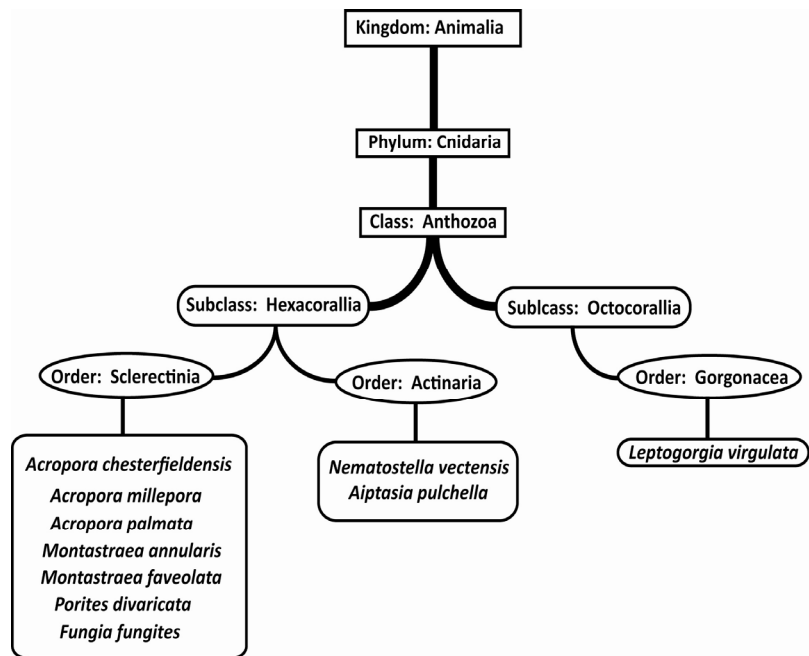


Figure 1. Simple Classification Scheme for Cnidarians presented in this document.

Coral reefs consist of many different cnidarian species with scleractinian (stony) coral providing the habitat framework for at least 25% of all marine species. They have been described as the most biologically diverse marine ecosystem on earth and analogous to tropical rainforests. Coral reefs also impart ecological services to humans including shoreline protection, natural products, bioceuticals and support local and national economies through fisheries, tourism, and recreation. Anthropogenic stressors (e.g., pollution, overfishing, increased coastal development and environmental changes) have negatively impacted coral reefs with 19% of reefs already lost. Further, predictions estimate an additional 15% loss over the next 10-20 years and another 20% loss in 20-40 years (Wilkinson 2008). Since the 1970's, Caribbean acroporid corals have declined as much as 90% (Raymundo *et al.* 2008) resulting in *Acropora palmata* (elkhorn coral) and *Acropora cervicornis* (staghorn coral) being listed in 2006 as threatened under the Endangered Species Act (ESA). The U.S. NOAA National Marine Fisheries Service (NMFS) is conducting a status review of an additional 82 candidate coral species for possible listing under the ESA (75 FR 6616). With the continuing global decline of reefs, it is critical to evaluate the health of corals and determine specific stressors affecting them to better support mitigation and protective management practices.

Cellular diagnostics is one approach to assess coral health. This approach takes advantage of assays that measure various cellular parameters (or biomarkers) as an indicator of biological state. Cellular diagnostics have been used to evaluate coral condition (Downs 2005) by recognizing that certain biomarker levels fluctuate in response to exogenous stressors and can be quantitatively measured.

Porphyrin levels are a key diagnostic endpoint in determining coral metabolic condition because they serve as prosthetic groups for an array of critical biochemical and cellular bioactive molecules involved in oxidative metabolism, respiration, antioxidant defenses, amino acid metabolism, fatty acid desaturation, and detoxification (Milgrom 1997; Krishnamurthy *et al.* 2007) (Fig. 2).

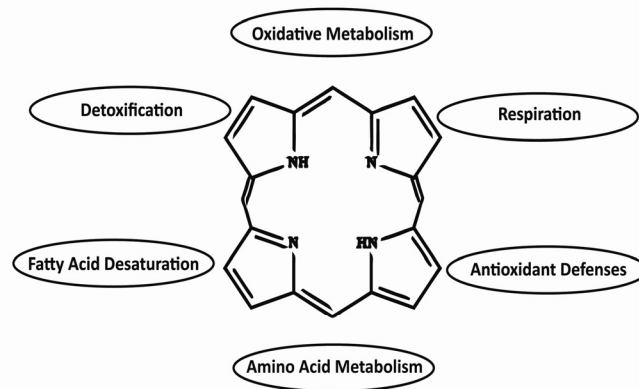


Figure 2. Porphyrins are Ubiquitous in Nature and Essential to Life.

Their diagnostic value in humans and animals primarily has been in helping to diagnose contaminant exposures (e.g., polycyclic aromatic hydrocarbons (PAHs)) (Marks 1985; Casini *et al.* 2003). However, their ubiquitous nature, diagnostic value, and susceptibility to contaminant exposures have given us confidence in using them as an indicator of cellular condition in coral (Downs *et al.* 2006; 2011).

Structure and Function of Porphyrins

Porphyryns are aromatic, heterocyclic macromolecules that have double-bonds whose names are derived from the Greek word *porphura*, meaning purple. Porphyrinogens are porphyrin precursors that lack double bonds and are colorless, but they are highly unstable and readily oxidize to porphyryns (Abe *et al.* 1989). Tetrapyrrolic porphyryns are comprised of four pyrrole rings connected by methine bridges, which can chelate various metals at their nitrogenous centers. Porphyrin serves as a prosthetic group for a variety of biochemical molecules (Fig. 3), e.g., cytochrome *c*, chlorophyll *a* and vitamin B12 with iron, magnesium, and cobalt chelated to their nitrogenous centers, respectively.

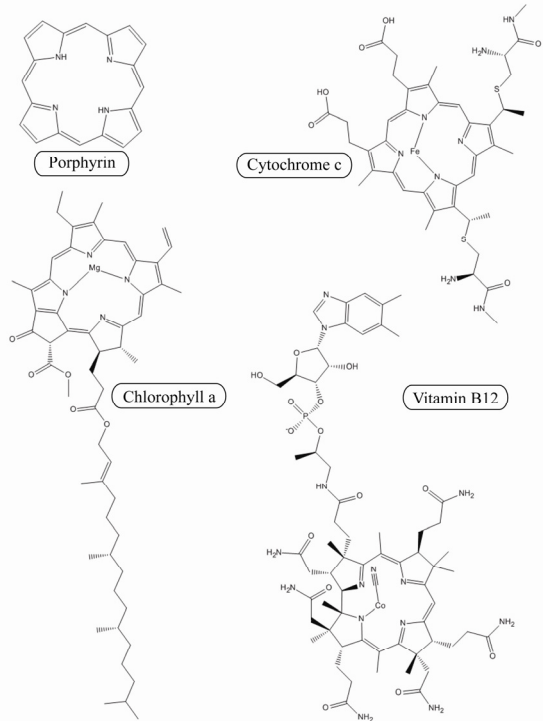


Figure 3. Porphyrin Ring Structures.

Porphyryn biosynthesis is part of the heme biosynthetic pathway. In animal cells, biosynthesis takes place within the mitochondria and cytosol while in plants, porphyryn biosynthesis also occurs in plastids (Fig. 4). Chlorophyll diverges from the pathway with the insertion of magnesium by magnesium chelatase, at the protoporphyrin IX intermediate.

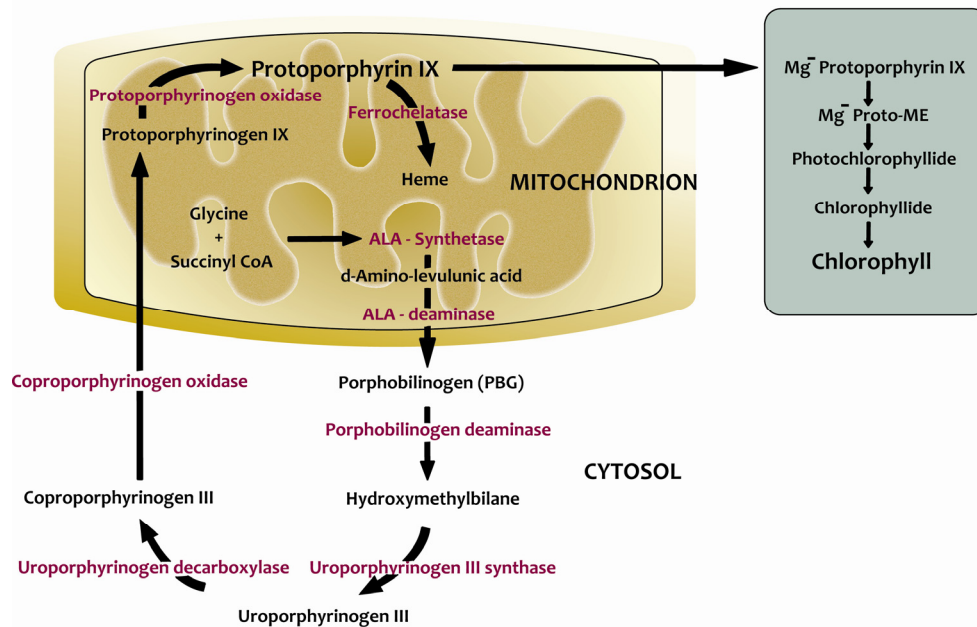


Figure 4. Porphyryn Biosynthesis.

There are eight key enzymes within the heme biosynthetic pathway that can be altered by heavy metal or organic chemical exposures. Disruptions in the pathway can prompt an over production of porphyrins and their intermediates. This can lead to the manifestation of porphyrias, a group of porphyrin metabolic disorders that can be genetically inherited. Other porphyrin disorders are acquired and develop from alterations in the heme biosynthetic pathway due to chemical exposures. Thus, changes in porphyrin levels can serve as an index of exposure to certain types of xenobiotics (Huuskonen *et al.* 1998; Marks 1985; Duke *et al.* 1991; Hahn 1996; 1997; Casini *et al.* 2003) (Table 1). The detection and quantification of porphyrin biochemical intermediates by standard clinical diagnostic methods have been primarily applied to humans (Kennedy *et al.* 1995; Muzyka *et al.* 2003; Deacon *et al.* 2008), terrestrial animals (Roscoe *et al.* 1979; Fox *et al.* 1988), and to a lesser extent, marine organisms (Hahn *et al.* 1996; Casini *et al.* 2006). Few studies have been conducted in marine invertebrates (Fossi *et al.* 2000; Koenig *et al.* 2009) including scleractinian coral (Downs *et al.* 2006; 2011).

Table 1. Chemically Induced Alterations in Heme Biosynthesis. Key enzymes affected by specific chemical exposures (Marks 1985; Daniell *et al.* 1997).

Enzyme Deficiency	Toxic Agents Affecting Heme Biosynthesis
ALA-Deaminase	Lead, Gold, Indium
Uroporphyrinogen III synthase	undetermined
Uroporphyrinogen decarboxylase	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC), Arsenic, Mercury, Cadmium, Hexachlorobenzene, 2,3,7,8-tetrachlorodibenzo-p-dioxi (TCDD), Polychlorinated biphenyl (PCB)
Porphobilinogen deaminase	Mercury, Cadmium
Protoporphyrinogen oxidase	Herbicides (p-nitrodiphenyl ethers, oxadiazoles, and cyclic imdides)
Coproporphyrinogen oxidase	Lead, Pesticides (diazinon, isadiazinon), Allylisopropylacetamide (AIA), Steroids
Ferrochelatase	Lead, Gold, Mercury, Cadmium, DDC
ALA-Synthase	Mercury, Cadmium, Indium, Cobalt, Hydrogen Sulfide, Methylmercaptan

Porphyrin Measurement

Classical methods for analyzing porphyrins in biological tissues have included paper chromatography (Nicholas *et al.* 1951; Chu *et al.* 1953), spectrophotometric detection (Rimington 1960; Muzyka *et al.* 2003), spectrofluorometric detection (Granick *et al.* 1972; Grandchamp *et al.* 1980; Valcàrcel *et al.* 1987; Westerlund *et al.* 1988; Kennedy *et al.* 1995; Fossi *et al.* 1996; Taylor *et al.* 2000; Koenig *et al.* 2009), and liquid chromatography (HPLC) (Lim *et al.* 1983; Abe *et al.* 1989; Perkins *et al.* 1989). Nicholas *et al.* (1951) and Chu *et al.* (1954) used one of the earliest qualitative methods, paper chromatography, for separation of porphyrin compounds from urine and fecal extracts (obtained from terrestrial animals and

human). Quantitative measurement of porphyrins became possible with the introduction of spectrophotometers, which capitalized on the spectral absorption of porphyrins in the Soret region (~400 nm), which is enhanced by acidifying the sample (Rimington 1960).

Spectrofluorometry is more sensitive and selective than earlier methods because it measures the intensity of fluorescence which is directly proportional to the intensity of excitation while spectrophotometry relies on the ratio of transmitted and absorbed light. The excitation intensity can be manipulated to generate increased fluorescence thereby increasing the sensitivity of the system. This method also capitalizes on the diagnostic Soret band (i.e., strong ultraviolet absorption band) of porphyrins. Using spectrofluorometry, Grandchamp *et al.* (1980) identified and quantified different porphyrins (uroporphyrin, coproporphyrin, and protoporphyrin) in cultured fibroblasts from patients with erythropoietic porphyria and coproporphyria based on their different emission spectra and differing excitation wavelengths. Muzyka *et al.* (2003) used spectrophotometric and spectrofluorometric detection to quantify protoporphyrin from workers exposed to diesel exhaust demonstrating the value of porphyrin measurements as a diagnostic marker of chemical exposure.

The spectrofluorometric method was improved further by Westerlund *et al.* (1988) who demonstrated that porphyrinogens must be completely oxidized to porphyrins within urine samples using hydrochloric acid and iodine to ensure accurate quantification. A further improvement to porphyrin analysis was the adaptation to a microplate fluorescence-based assay. Kennedy *et al.* (1995) used chick embryo hepatocyte cultures exposed to polychlorinated biphenyls (PCBs) to validate the microplate assay by comparing it to HPLC analysis of the same samples. Even though total porphyrin concentrations were 15-20% higher in the HPLC purified samples, the authors considered their new method to be comparable for total porphyrin determination. However, HPLC is still preferentially used to accurately separate, identify, and quantify individual porphyrin species within biological matrices because other methods are unable to separate individual porphyrins in a mixture (Lim *et al.* 1983). Although HPLC separates porphyrin species, spectrofluorometry is suitable for total porphyrin quantification, which also is valuable in diagnostic and screening procedures at lower cost and higher throughput. Further it has the advantage of compatibility with samples derived from SDS and DTT based protein extraction methods.

Organic extraction is the classical method of releasing porphyrins from biological tissues and fluids. Sample preparation generally involves a solvent (e.g., diethyl ether, ethyl acetate or methanol) followed by acidification with hydrochloric acid (HCl) (Lim *et al.* 1982; Westerlund *et al.* 1988; Woods *et al.* 1993; Taylor *et al.* 2000; Casini *et al.* 2003). The classical organic extraction is not compatible with protein-based assays that use SDS and DTT-containing denaturing buffers (e.g., western blot, ELISA). The extraction method presented herein uses a denaturing buffer that is compatible across multiple protein-based assays allowing for different analyses from a single sample. An important modification to this assay is the use of a narrow bandwidth filter to minimize interference from chlorophyll *a* and *c* from the coral's symbiont (zooxanthellae) which can confound results if wavelengths above 620 nm are not cut off when samples are excited at 410 nm.

Porphyrin Method Overview

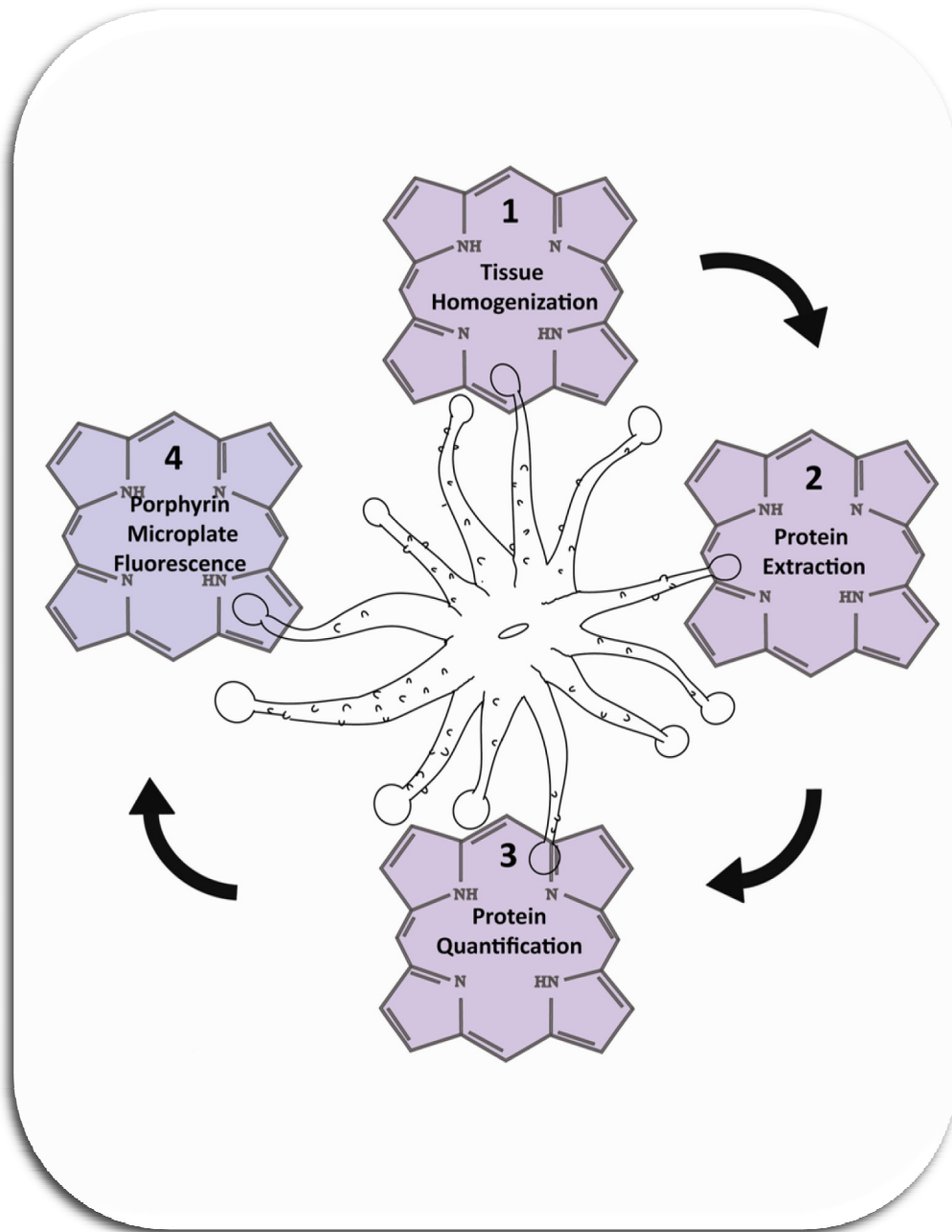


Figure 5. Method Overview

Method Overview

The modified porphyrin detection method described here requires four different protocols: 1) tissue homogenization, 2) denaturing protein extraction, 3) protein quantification, and 4) acidification of extracts, prior to porphyrin detection (Fig. 5). The protein homogenization and extraction procedures are modifications from Laemmli (1970) as described by Downs (2005) for coral tissue. The use of soluble protein extracts for porphyrin quantification based on these extraction procedures was reported first in Downs *et al.* (2006), while this document is the first to report the detailed procedures of the method in its entirety; validation results demonstrating the buffer components do not interfere with porphyrin detection; and includes examples of porphyrin levels in a variety of cnidarians species to demonstrate the method and range of porphyrin levels that may be encountered using this method.

Comprehensive Equipment & Supplies:

- 96-well optical bottom black plates (Fisher Scientific)
- 0.5 mL microcentrifuge tubes (USA Scientific, Ocala FL)
- 1.5 mL amber microcentrifuge tubes (USA Scientific)
- 1.5 mL Eppendorf microcentrifuge safe-lock natural tubes (Sigma)
- 2.0 mL cryovials, e.g., Corning (Fisher Scientific)
- Air blow dryer
- Bench paper (e.g., Versi-Dry® lab soakers, VWR International, Sugarland TX)
- Ceramic mortar & pestle (e.g., cat# 60313) chemical-porcelain mortar with 80 mm diameter, 53 mm height, 65 mL capacity and 130 mm length pestle (Coorstek, Golden CO)
- Micro sample tube pestle to fit in 1.5 mL tube
- Cutting board
- Digital imaging instrument with at least 0.3 – 1.2 megapixels (e.g., CCD camera, scanner, or G-Box®- with analysis software, Syngene, Frederick, MD)
- Emission filter 600 nm ± 20 emission filter (Biotek®, Winooski VT)
- Excitation filter 400 nm ± 15 (Biotek®)
- Freezer grip (e.g., Kevlar w/Latex Coated Palm Gloves, Wells Lamont, Fisher Scientific)
- Freezer mill for bulk grinding with grinding accessories (e.g., Freezer/Mill 6850, SPEX CertiPrep®, Metuchen NJ)
- Fume hood (e.g., Mott manufacturing, Ontario CA)
- Grinding equipment and accessories (grinding vial, impactors, end plug and extractor, 6850 Freezer/Mill SPEX CertiPrep®)
- Hammer & chisel (e.g., slot-head micro-screwdriver)
- Hemostat – approx. 8 inches
- Imaging software (e.g., Adobe Photoshop, NIH Image J or Genetools®- analysis software for GeneSnap®-Syngene)
- Liquid nitrogen (LN₂) and dewar (e.g., 4150 Dewar Flasks (Fisher Scientific)
- Microcentrifuge benchtop (24 x 1.5 mL / 2.0 ml tubes), 15,000 rpm, 21,000 x g (e.g., Micromax RF - Fisher Scientific)
- Micropipettors with 20-200 µL range (P200) and 0.5-2.0 µl range (P2) (e.g., Gilson Inc, Middleton WI)
- Minicentrifuge (6 x 1.5 mL rotor with 0.5ml adaptors), 6000 rpm, 2000 x g (rcf) (e.g., ISC BioExpress, Kaysville UT)
- Plastic container with lid (greater than 150 mm diameter)

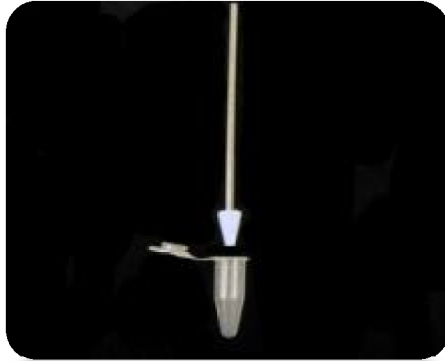
- Platform rocker with variable speed and steep angle rocking (e.g., Bellco Biotechnology, Vineland NJ)
- Scanning spectrofluorometer with λ 250-850 nm excitation range, λ 360-850 nm emission range, 96-well plate capacity, sensitivity at least 3.0 fmol/well with fluorescein with accompanying analysis software (e.g., SpectraMax Gemini XS Microplate spectrofluorometer, Molecular Devices Co., Sunnyvale CA)
- Spatulas (e.g., cat# 2140115; 5.5 in, blade 19 x 4.8 mm – Fisher Scientific)
- Spectrofluorometer with 200 nm–700 nm wavelength range, bandpass filter dependent, photomultiplier detection, 96-well plate capacity, and sensitivity of 1.0 fmol/well with fluorescein with accompanying analysis software (e.g., Synergy HT -Biotek®)
- Styrofoam™ container 6x8 inches with dividers to chill spatulas and cryovials with LN₂
- Thermometer – partial immersion 20-110°C (Fisher Scientific)
- Vortex mixer
- Water bath (approx. 6x10 inch inner dimensions) with heating range to 90°C
- Weigh boats (Fisher Scientific)
- Whatman No. 5 filter paper (e.g., 90 mm to 150 mm diameter- Fisher)

Comprehensive Reagents and Solutions List

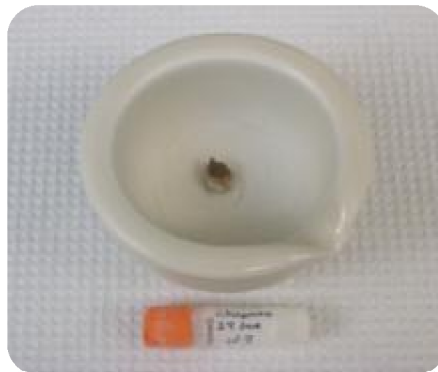
*The companies identified are those used in preparing this document. There are other suitable sources for many of these reagents; the important factor is to obtain fresh reagents, so always ask for the manufacturing date when purchasing reagents for this assay.

- 6-aminocaproic acid 99+% (Acros Organics, Geel, Belgium)
- Benzamidine hydrochloride hydrate (Acros)
- **BSA** - bovine plasma albumin protein standard (Bio-rad, Hercules CA)
- Coomassie Brilliant Blue R-250 (Sigma Aldrich, St. Louis MO)
- **CP III** - coproporphyrin III tetramethyl ester - cat# C710-3 (Frontier Scientific, Logan UT)
- **DM** - deferoxamine mesylate salt (Sigma)
- **DMSO** - dimethyl sulfoxide 99.9% ACS spectrophotometric grade - (Sigma)
- **DTT** - Dithiothreitol (Gold Biotechnology Inc., St. Louis MO)
- **EDTA** - ethylenediaminetetraacetic acid disodium salt dehydrate (Amersham Biosciences –GE Healthcare, Piscataway NJ)
- Glacial acetic acid A.C.S Plus (Fisher Scientific, Pittsburgh PA)
- **HCl** - concentrated hydrochloric acid technical grade (12N) (Fisher Scientific)
- Methanol sequencing grade (Fisher)
- **PMSF** - Phenylmethanesulphonylfluoride (Sigma)
- **PVPP** - Polyvinylpolypyrrolidone (Sigma)
- Protease inhibitor cocktail for plants cat# p9599 (Sigma)
- **PP IX** - protoporphyrin IX cat# P562-9 - (Frontier Scientific)
- **SDS** - sodium dodecyl sulfate - (EMD Biosciences, Inc. Darmstadt, Germany)
- Sorbitol (Fisher)
- **Tris** - tris (hydroxymethyl)aminomethane base - (Amersham)
- **URO III** - uroporphyrin III dihydrochloride catalogue # U830-3 - (Frontier Scientific)

Protocol 1: Tissue Homogenization



Soft Tissue Homogenization



Frozen Tissue Homogenization Using a Mortar & Pestle



Frozen Tissue Homogenization Using a Freezer Mill

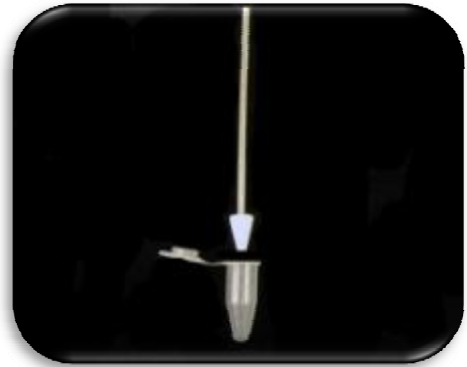
Figure 6. Protocol 1 Overview

Protocol 1: Tissue Homogenization

A. Soft Tissue Homogenization

Supply Checklist

- Denaturing Buffer
- Micro sample tube pestle
- Transfer pipette
- P200 micropipette & tips
- 1.5 mL safe-lock microcentrifuge tubes



Procedure:

1. Remove as much moisture from the tissue samples as possible. Suction off extra liquid and mucus using a transfer pipette for small tissue samples (e.g., *N. vectensis*, *Aiptasia*, or soft corals).
2. Add 200 μ L of denaturing buffer and tissue to graduated 1.5 mL safe-lock microcentrifuge tubes.
3. Homogenize fresh samples with a conical shaped tissue homogenizer sized for 1.5 mL microcentrifuge tubes.
4. Add the rest of denaturing buffer to the homogenized sample 100 μ L at a time and homogenize each time as needed until volume has reached (400-1000 μ L) depending on the amount of the sample (e.g., 50-170 mg of *N. vectensis*).
5. Homogenized samples are ready for protein extraction.

B. Frozen Tissue Homogenization Using a Mortar and Pestle

Supplies for Homogenizing Frozen Tissues using Mortar and Pestle (Fig. 7)

- Hammer & chisel
- Cutting board
- 2 mL cryovials
- Freezer grip or insulated glove
- Insulated container with dividers for LN₂
- Spatulas
- Weigh boats
- LN₂ & LN₂ Dewar
- Hemostat
- Mortar & pestle (for pieces < 2cm)



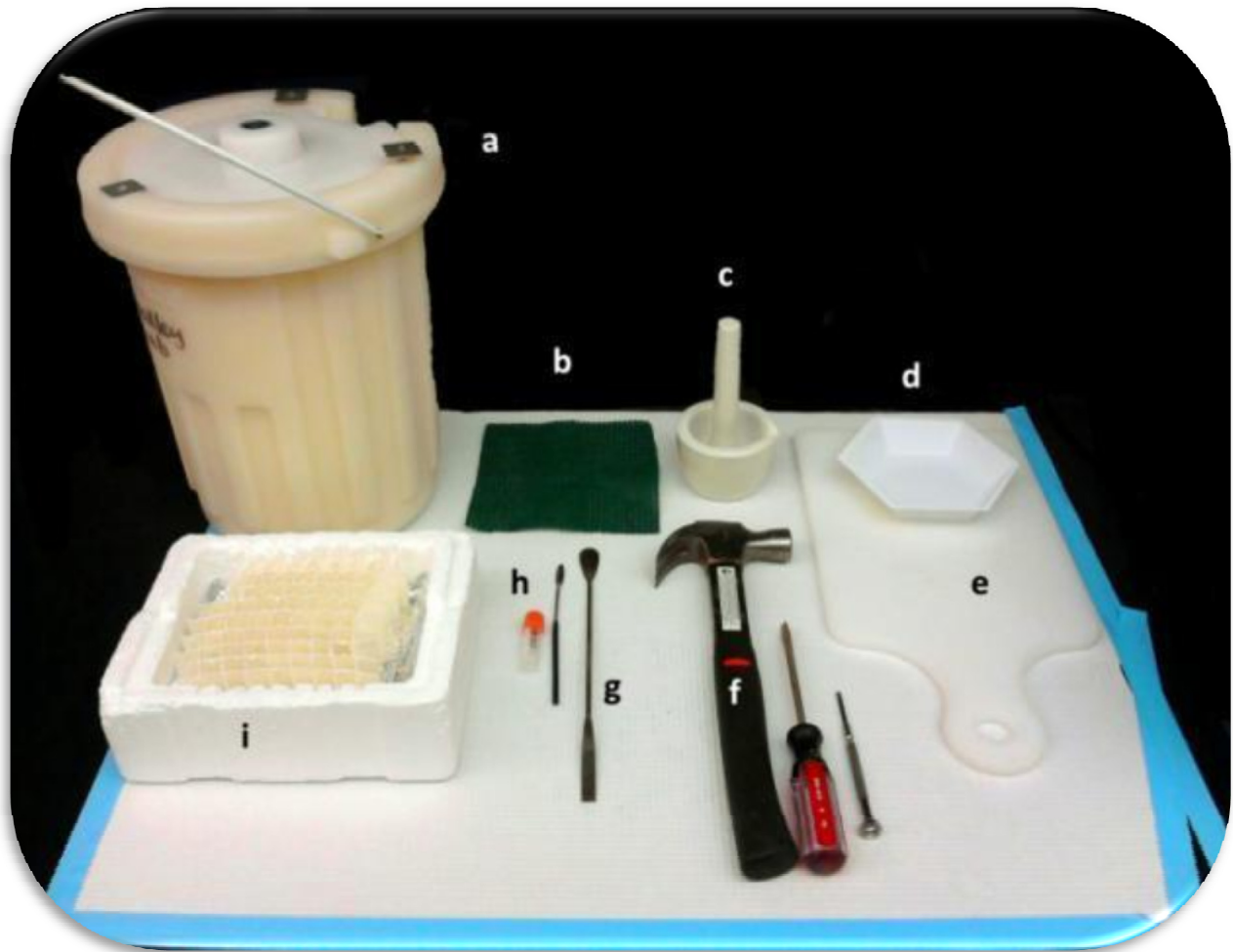


Figure 7. Grinding Materials a) Dewar, b) freezer grip, c) mortar and pestle, d) weigh boat, e) cutting board, f) hammer and chisels, g) spatulas, h) cryovial, and i) Styrofoam™ box with dividers.

Procedure:

1. Cover work area with absorbent pad (bench paper) and chill cleaned spatulas in LN₂.
2. Use a clean hammer and chisel rinsed with 70% ethanol to remove visible algae and excess skeleton from coral fragments before grinding.
3. Set clean pestle upright within the clean mortar and pre-chill by filling with LN₂ and letting it evaporate twice before adding tissue sample.
4. Add tissue fragments to mortar and fill halfway with LN₂. Let the LN₂ evaporate before commencing grinding. *Pouring too quickly can cause the tissue to slosh out of the mortar from the bubbling LN₂.*
5. Using an insulated rubber grip (freezer grip) to hold mortar, slowly grind while using the gloved hand that holds the mortar to partially cover the top of the mortar. Change gloves between samples to avoid cross contamination.
6. Intermittently add LN₂ to prevent thawing and to allow ease of grinding tissue.
7. Correctly ground tissue should be homogeneous and the consistence of flour.

8. Use pre-chilled spatulas to dispense ground tissue into 2 mL cryovials. *Do not close tubes until LN₂ has evaporated to avoid pressure build up from the nitrogen gas.*
9. Store ground tissue samples at -80°C. For long-term storage consider a liquid nitrogen freezer.

C. Frozen Tissue Homogenization Using a Freezer Mill

Supplies for Homogenizing Frozen Tissues using Freezer Mill (Fig. 8)

- Freezer Mill
- Grinding vials
- Impactors
- End plugs
- Extractor
- See list in section B above for additional supplies



Procedure:

1. Chill freezer mill with LN₂ according to the manufacturer's recommendation.
2. Number grinding vials and keep a log of numbers in relation to sample information. Pre-label all cryovials for storing ground tissue.
3. Insert a tube stand to hold grinding vials in a Styrofoam™ container filled with LN₂ covering the lower ¾ of the vials.
4. Insert stainless steel end plugs into the end of grinding vials, add steel impactors to the vial and chill in LN₂ (Fig. 8). *Do not chill more vials than the mill holds at a time because frost build-up can interfere with sample integrity.*
5. Using a separate insulated container (e.g. Styrofoam™ container) filled with LN₂, insert dividers (e.g., from freezer boxes) along with pre-labeled 2 mL cryovials for ground tissues and chill enough spatulas for each sample. Select appropriate sized cryovials (range 1.2 mL to 5 mL) for varying amounts of tissue.
6. Prior to grinding a sample, place it onto a clean surface (e.g., weigh boat) and inspect each piece for endolithic (in the skeleton) algae or other contaminating epibionts (surface). Photo-document samples, if required then remove the algae, epibionts and excess skeleton using a hammer and chisel or slot-head micro-screwdriver, ensuring the tissue remains frozen by adding LN₂ as needed. The goal is to obtain as much tissue

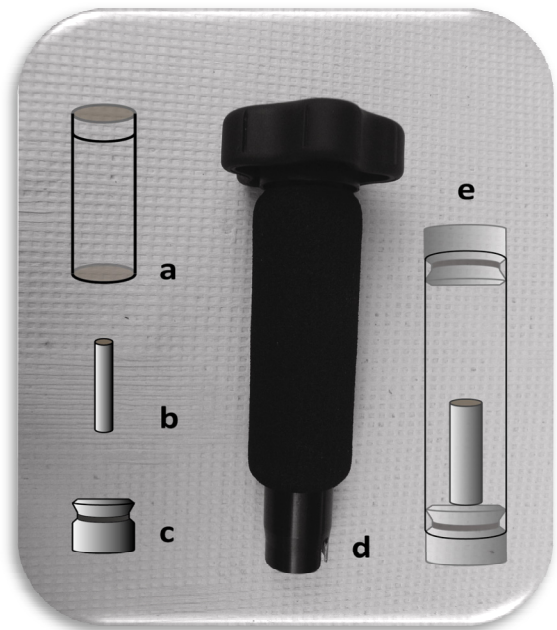


Figure 8. Grinding Accessories: a) grinding vial, b) impactor, c) end plug, d) extractor, and e) assembled sample vial.

(polyp depth) possible without extraneous matter for the cleanest and most concentrated samples. Larger coral pieces require fragmenting to easily fit into grinding vials.

7. Place frozen tissue fragments into chilled grinding vials, making sure fragments fit loosely around the steel impactor. Do not exceed the amount of tissue recommended by the manufacturer.
8. Experimentally determine the parameters for milling tissue using the manufacturer's guidelines for grinding, duration, and impact frequency.
9. Follow manufacturer recommendations for retrieving samples from the freezer mill.
10. Open each grinding vial using the extractor tool and inspect the consistency of the ground tissue. The tissue should be ground homogeneously to the consistency of flour.
11. Use a clean pre-chilled spatula to scoop ground tissue into chilled cryovials and place them into LN₂ prior to storage at -80°C.

Time Considerations

The freezer mill operation for 25 stony coral samples, will take 2 to 5 hr with a minimum team of two people. Most of the time is spent removing extraneous endolithic algae and excess skeleton from coral samples prior to grinding. Grinding 20 samples with a mortar and pestle can take up to two 8 hr days depending on the size of the coral fragment and the amount of endolithic algae associated with the samples.

Sample Considerations

Homogenization with the freezer mill can be used for all frozen tissue samples that are in large quantity and meet the size limit recommended by the manufacturer and optimal for more than 20 samples that are 2 cm or greater. Refer to the manufacturer's recommendation for sample size limitations and instrument operation.

Safety Considerations

Care should be taken when handling liquid nitrogen to guard against severe frostbite from dermal contact, asphyxiation from vapors when concentrations are high enough to reduce oxygen levels and physical harm from exploding cryovials when liquid nitrogen is trapped in cryovials. Review material safety data sheet (MSDS)

Protocol 2: Protein Extraction

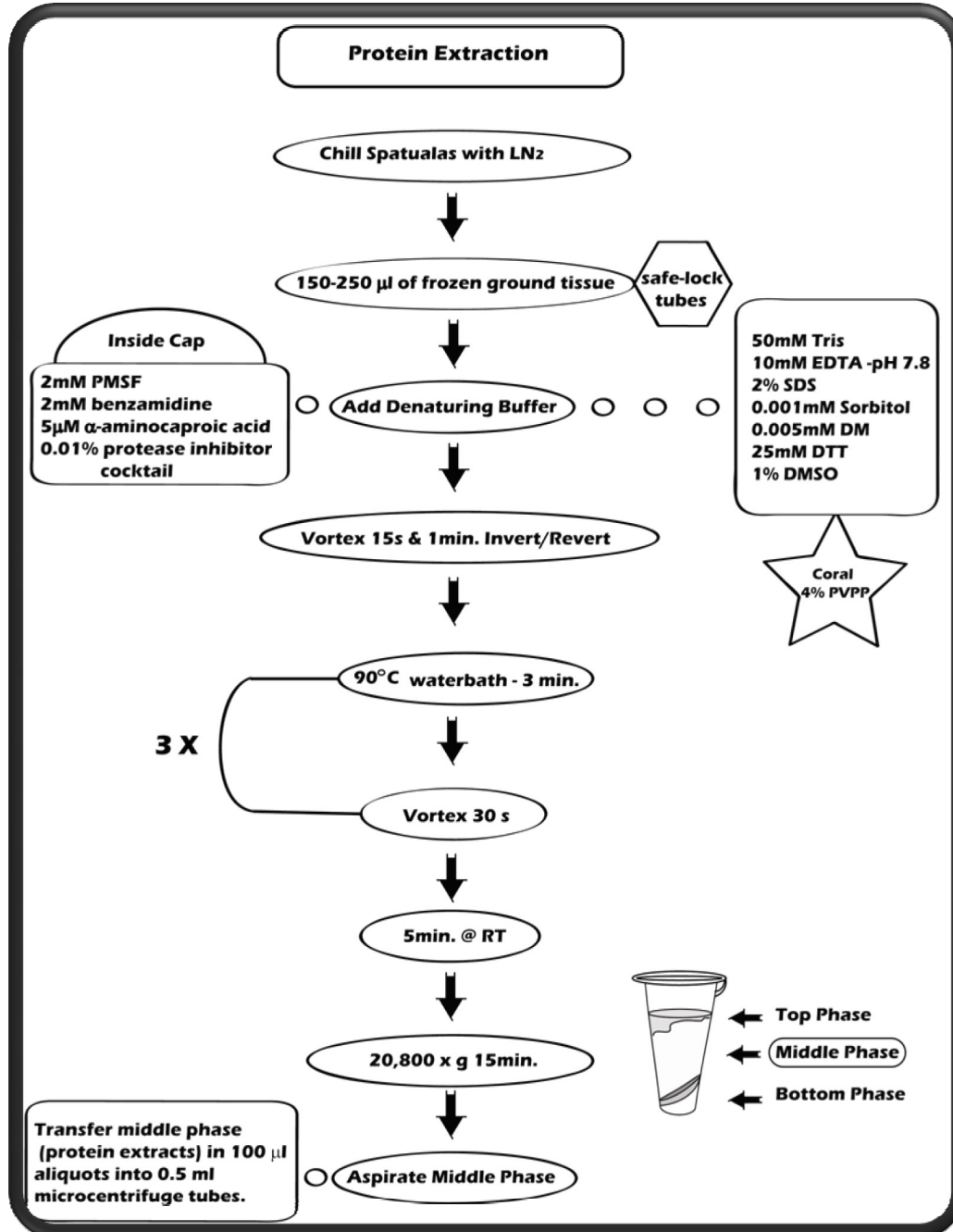


Figure 9. Protein Extraction Flowchart

Protocol 2: Protein Extraction

Equipment & Supplies Checklist

- 1.5 mL amber tubes
- 1.5 mL safe-lock microcentrifuge tubes. *Locking-cap microcentrifuge tubes prevent caps from popping open when samples are heated.*
- 2.0 mL cryovials containing frozen ground samples
- Denaturing buffer
- Freezer grip or insulated glove
- Hemostat
- LN₂ (Dry ice may be used as a substitute) & dewar
- Microfuge with maximum *g* force 21,000
- Micropipettors with 20 – 200 µL range (P200) and 100 – 1000 µL range (P1000) and tips
- Spatulas
- Styrofoam™ container with tube inserts
- Thermometer (partial immersion - 0-110°C)
- Tube rack
- Vortex mixer
- Water bath set to 90°C to hold up to twelve 1.5 mL safe-lock tubes

Reagent List & Preparation

Stock Solutions

- 1 mM sorbitol (0.182 g/L in dH₂O)
- 5 mM 6-aminocaproic acid (0.656 g/L in dH₂O)
- 50 mM DM (32.8 g/L in dH₂O)
- 200 mM benzamidine (31.3 g/L in dH₂O)
- 200 mM PMSF (34.8 g/L in 100% ethanol)

Tris-EDTA-Sodium Dodecyl Sulfate (TE-SDS) Buffer

- 50 mM Tris
- 10 mM EDTA (pH is adjusted to 7.8 with HCl after EDTA is mixed with Tris)
- 2% SDS (add after pH is adjusted to 7.8)

Denaturing Buffer Part 1 (DB1)

- 50 mM Tris
- 10 mM EDTA (pH is adjusted to 7.8 with HCl after EDTA is mixed with Tris)
- 2% SDS (add after pH is adjusted to 7.8)
- 0.001 mM Sorbitol
- 0.005 mM DM
- 25 mM DTT
- 1% DMSO
- 4% PVPP

Denaturing Buffer Part 2

- Protease inhibitors (added separately into microcentrifuge cap):
- 5 μ M 6-aminocaproic acid
- 2 mM benzamidine
- 2 mM PMSF
- 0.01% protease inhibitor cocktail for plants.

Prepare 20 mL of DB1 for up to 15 samples. Weigh 121.14 mg of Tris base and 74.4 mg of EDTA, mix with 16 ml of deionized/distilled H₂O in a 50 mL conical tube. Adjust the pH of the Tris-EDTA solution to pH7.8 using 1N HCl, then add 4 g of SDS (SDS concentration needs to be experimentally determined for each species). Do not vortex the TE-SDS solution; instead swirl solution until SDS is solubilized. Warming the solution will help bring the SDS into solution. Add 20 μ L of 1 mM of sorbitol, 20 μ L of 50 mM DM, 77.1 mg DTT, 200 μ L DMSO and bring to a final volume of 20 mL with dH₂O. Finally, add 0.8 g of PVPP to the denaturing buffer. Note that PVPP is insoluble and will remain as a suspension in the buffer. Gently swirl denaturing buffer each time before adding to tissue to ensure PVPP is in suspension and distributed evenly among samples.

Protease inhibitors are kept separate from DB1 in the inside cap of open pre-labeled 1.5 mL safe-lock microcentrifuge tubes. The following quantities are added to the cap: 1 μ L of 5 mM 6-aminocaproic acid, 10 μ L of 200 mM benzamidine, 10 μ L of 200 mM PMSF, and 20 μ L of 0.01% protease inhibitor cocktail (stored in 50% glycerol). Do not close caps.

Procedure:

1. Label 1.5 mL safe-lock microcentrifuge tubes.
2. Prepare fresh denaturing buffer at the beginning of each extraction day.
3. Transfer cryovials containing ground tissue from -80°C into LN₂ or dry ice until used.
4. Chill spatulas in LN₂ before dispensing ground tissue to prevent tissue from thawing or adhering to spatulas, next retrieve one tissue vial from the LN₂ using a hemostat.
5. Add protease inhibitors to the inside caps of a set of four (4) open, pre-labeled safe-lock microcentrifuge tubes. *Do not close caps.*
6. Transfer 150 to 250 μ L of frozen ground tissue sample using a chilled spatula into the first graduated 1.5 mL safe-lock microcentrifuge tube. Immediately add 1 mL of denaturing buffer and close cap to introduce protease inhibitors. In scleractinians, the tissue to skeleton ratio varies for each species and must be taken into consideration when optimizing tissue to buffer ratios for each set of samples. *See troubleshooting and optimization section below.*
7. Vortex the sample tube for 30 s and set aside at room temperature.
8. Repeat steps 6 & 7 for the next 3 samples in the set. [Recommend processing samples in small batches to allow the user to move through the denaturing steps quickly without compromising the sample integrity. The user will have to determine how many samples can be handled to maintain the timing requirements of the procedure.]
9. Next gently invert the 4 sample tubes repeatedly for 1 min for the denaturing buffer to completely interface and mix with the sample.

10. Incubate samples in a 90°C water bath for 3 min.
11. Retrieve samples from the water bath and vortex for 30 s.
12. Repeat steps 10 and 11 twice and place tubes at room temperature (23-25°C) for 5 min.
13. Spin samples for 15 min at 20,800 x *g*. Three phases should be visible after centrifugation (see Fig. 9).
14. Carefully aspirate the middle phase of sample extract using a micropipette with a 20-200 µL range taking care not to allow the pipette tip to touch the inside of the tube, thus minimizing the chance of contamination from other phases. The middle phase should contain the soluble protein fraction free of mucus and insoluble particles. *Change the micropipette tip if an additional aspiration is needed, to ensure that the mucus adhering to the tip from the top phase is not carried over into your sample.*
15. Aliquot a sufficient amount (50 – 100 µL) of protein extract per 0.5 mL tube to do one assay. Store protein extracts at -80°C. Avoid repeated freeze/thaw of extracts to prevent protein degradation and to maintain protein stability.
16. Prior to freezing the protein extracts at -80°C, remove 3 µL from each protein extract for protein quantification as described in the next section.

Troubleshooting and Optimization

1. It is imperative to optimize protein extraction procedures for each species because the ratio of skeleton to tissue mass and the amount of mucus will vary among species. The ratio of skeleton to tissue mass will determine the ratio of denaturing buffer to ground tissue. Similarly species differ in the amount of mucus they produce and this method will require experimental optimization to minimize mucus contamination. A good indicator of protein yield due to the proper ratio is the color of the middle phase after centrifugation. A good indication of low protein yield is a very pale yellow to clear color. As the color of extract gets darker, protein yield increases.
2. Centrifugation parameters in Downs' (2005) protocol were insufficient to yield samples suitable for western blot and ELISA analysis for the species tested (*Acropora cervicornis*, *Acropora chesterfieldensis*, *Acropora palmata*, *Montastraea annularis*, *M. faveolata*, *Oculina varicosa*, *Porites astreoides*, *P. divaricata*, and *P. lobata*). However, by increasing the centrifugal force from 13,000 x *g* to 20,800 x *g*, optimal separation of the three phases was achieved. Prior to this adjustment, the top layer readily fell into the middle phase before aspiration. The centrifugal (*g*) force can be adjusted and/or multiple centrifugations steps may be needed for species that have more mucus such as *Montastraea* and *Oculina*.
3. The three visible phases associated with scleractinian coral extraction may not be as obvious in other cnidarians such as *N. vectensis*. Since *N. vectensis* has minimal amounts of mucus and no skeleton, the three phases are not visible. See Fig. 9 for an illustration of the three phases.
4. Maximum protein yield was attained after including an additional step (Step 9 pg. 16) of gently inverting the sample repeatedly for 1 min, to completely mix the tissue with denaturing buffer. This was based on observations that the tissue did not disperse evenly by solely vortex mixing; hence, the tissues did not come into complete contact with the denaturing buffer or protease inhibitors.

5. Tissue samples can be lost when removing cryovials from LN₂ if care is not taken. Liquid nitrogen can leak into the cryovials causing the caps to pop off and tissue to erupt from the vial. To prevent sample loss, LN₂ must completely evaporate before scooping ground tissue into cryovials. Make sure that the caps are tightened. This can also be an issue when using the freezer mill. Another cause for sample loss is using gloved hands without enough insulation to protect the vials from warming up in your hands when opening the caps and scooping out sample for protein extraction. Body heat will warm up the vials quickly when the cap is removed and may result in loss of precious coral tissue.
6. The porphyrin assay can tolerate slight mucus contamination so material from the top or middle phase can be used. Most other assays, require clean extracts (middle phase only) free of mucus (e.g., western blot and ELISA).

Time Considerations

Preparation of buffers and extracting protein from 20 samples may take approximately 2.5 hr.

Denaturing Buffer Considerations

The denaturing buffer composition should be tailored to meet the needs of the assay that the extracted protein will be used in:

- EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate) is a metal chelator.
- PVPP (polyvinylpyrrolidone) is used to bind polyphenolic compounds found in cnidarian samples that contain symbiotic algae (zooxanthellae). This is an insoluble compound that should be removed during the centrifugation process.
- SDS is an anionic detergent used to facilitate the denaturation (unfolding) of proteins by disrupting their non-covalent bonds. This component requires optimization for each species and typically can range from 2-4%.
- Sorbitol is a sugar alcohol used as a cryoprotectant.
- DM is an iron chelating agent to prevent Mallard products.
- DTT is a reducing agent that breaks disulfide bonds of proteins. For disulfide bonds that are covered due to the folding nature of the protein, heat and or a stronger denaturant (e.g., Urea, SDS, and guanidium hydrochloride) can be used with DTT.
- DMSO is an organosulfur solvent that facilitates protein solubilization and phase partitioning of the mucus.
- Protease inhibitors target specific *protease classes*:
 - 5 μM 6-aminocaproic acid: *Lysine proteases*
 - 2 mM benzamidine: *trypsin and serine proteases*
 - 2 mM PMSF: *serine proteases*
 - 0.01% protease inhibitor cocktail: *serine, cysteine, aspartic, metalloproteases, and aminopeptidases.*

Protocol 3: Protein Quantification

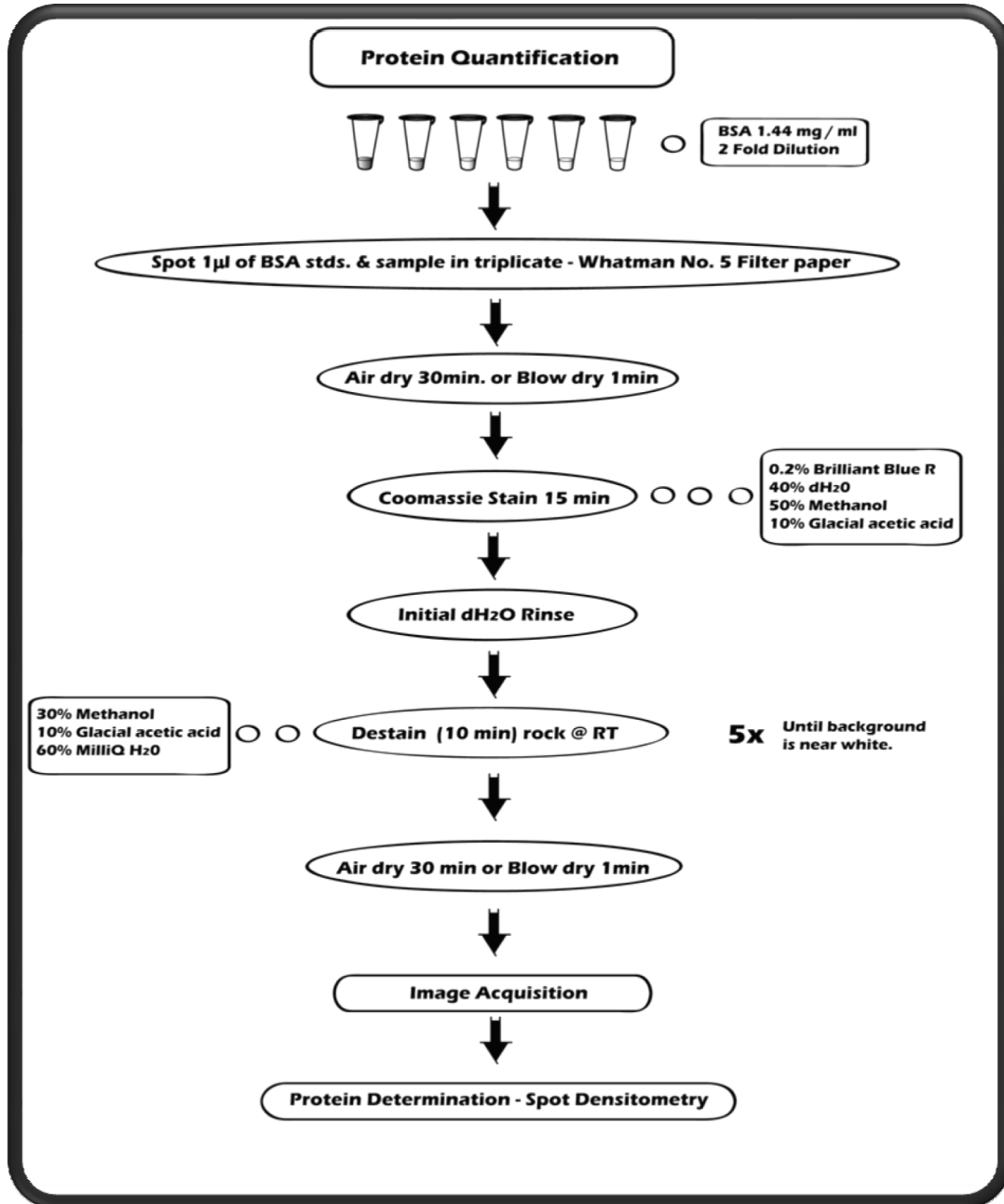


Figure 10. Protein Quantification Flowchart

Protocol 3: Protein Quantification

A. Spot Blot

Equipment & Supplies Checklist

- Air blow dryer
- BSA
- Micropipettor with 0.5 – 2.0 μL range (P2) and tips
- No. 2 pencil
- Plastic container with top, no smaller than 150 mm diameter
- Rocking platform
- Tube rack
- Vortex mixer
- Water bath set to 65°C (minimum capacity of 12 microcentrifuge tubes)
- Whatman No. 5 filter paper 90 mm to 150 mm in diameter

Reagent List & Preparation

TE-SDS

- 50 mM Tris
- 10 mM EDTA (pH is adjusted to 7.8 with HCl after EDTA is mixed with Tris)
- 2% SDS (add after pH is adjusted to 7.8)

Coomassie Stain

- 0.2% Brilliant Blue R-250
- 40% Deionized/distilled water (ddH₂O)
- 50% Methanol
- 10% Glacial acetic acid

Coomassie Destain

- 30% Methanol
- 10% Glacial acetic acid
- 60% ddH₂O

Prepare 0.5 L of Coomassie stain for up to 20 staining incubations (50 mL per 150 mm spot blot used twice). Weigh 1 g of brilliant blue R-250 (dye) and dissolve in 250 mL of 100% methanol, 50 mL of glacial acetic acid, and bring volume to 500 mL with ddH₂O. Stir solution until stain is fully dissolved. Filter the solution through Whatman No. 5 filter paper. Next, prepare 2.0 L of destain for up to 8 spot blots (150 mm diameter). Pour 1.2 L of ddH₂O into glass container, then add 600 mL of 100% methanol, and finally 200 mL of glacial acetic acid to complete the destain solution (*always add acid to water and not the reverse*). Lastly, make up 20 mL of TE-SDS. Weigh 121.14 mg of Tris base and 74.4 mg of EDTA, mix with 16 mL of ddH₂O in a 50 mL conical tube. Adjust the pH of the Tris-EDTA solution to pH7.8 using 1N HCl. Finally, add 4 g of SDS, swirl (**do not vortex**) and warm solution until SDS is solubilized. (**Save remaining solution for Protocol 4**).

Procedure:

1. Prepare a six-point calibrant curve using 2-fold dilutions (1.44 mg/mL – 0.045 mg/mL) from 2.88 mg/mL dissolved BSA in TE-SDS. *This range can be modified as needed.*
2. Heat BSA standard dilutions and frozen sample extracts to 65°C for 5 min and spin for 10 sec at 2000 x g and then vortex for 15 sec.
3. Pipette 1 µL of each BSA standard and sample in triplicate and uniformly onto Whatman No. 5 circular paper (see Fig. 11). Under each column of triplicate spots, draw a line with a no. 2 pencil to label the sample and provide a reference point. This helps to avoid overlapping application of extracts onto those that have dried and are no longer visible.
4. Air dry spot blot for 30 min or dry with an air blow dryer in less than 1 min.
5. Place spot blot into Coomassie stain in a plastic container with lid for 15 min on a reciprocal rocking platform at ambient temperature.
6. Decant stain and rinse excess stain from the spot blot with a quick flush of water. Do not pour water directly onto the spot blot.
7. Incubate spot blot in destain for 10 min rocking at room temperature. Decant and repeat incubation with fresh destain four more times for a total of 50 min or until background is near white.
8. Dry blot (see step 4).

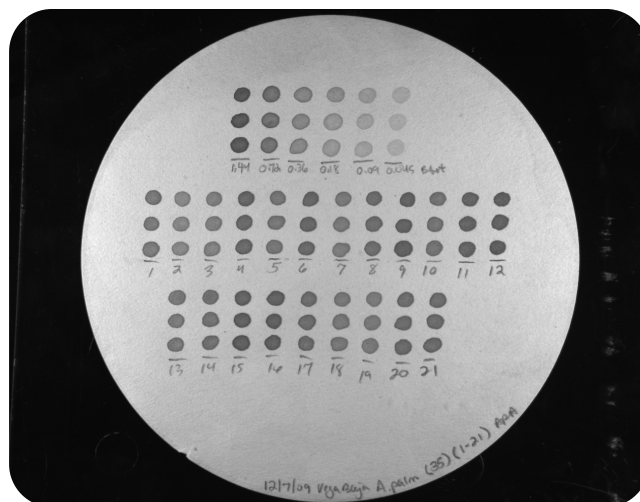


Figure 11. Spot Blot of *Acropora palmata* Protein Extracts. BSA standards range from 1.44 µg/µL to 0.045 µg/µL shown at the top of the blot in triplicate, followed by 21 protein extracts in triplicate. The spots are uniform and within the standard BSA range.

B. Spot Densitometry

Equipment & Supplies Checklist

- Digital imaging instrument
- Imaging software
- Imaging analysis software

Procedure: Image acquisition using an imaging system (e.g., Syngene G-Box®)

1. Place spot blot within the G-Box unit onto the gel box.
2. Place a clean glass plate on top of the spot blot to keep it from curling.
3. Adjust the parameters (iris, zoom, fine focus, brightness, contrast, and gamma) using the software provided by the imaging system (e.g., GeneSnap®).

4. Select “upper-white” and “no binning” before acquiring the image according to the manual’s instructions.
5. Save both sgd file format and TIFF files (.sgd to be used in Genetools® and Tagged Image File Format to be opened using most imaging software).

Note: Alternatively, a digital scanner may be used to acquire a grayscale image of the spot blot. The image file should be saved as a TIFF to be analyzed later. Alternative programs used to calculate the density of protein spots are discussed below.

C. Protein Determination

Procedure: *Imaging System analysis software (e.g., GeneTools®)*

1. Select the following options under the “General” tab: “Spot Blot, Circle, Absorption, and Leave spots unchanged”. Select “OK”.
2. Edit “Quantity Calibration” parameters by selecting curve type and give the units ($\mu\text{g}/\mu\text{L}$).
3. Manually insert the first trace circle around the first BSA standard spot 1 by double clicking on the center of this spot. Select “OK”.
4. Adjust the area of the trace circle by dragging the outer edges of the circle without extending over the perimeter of the circle. To move the trace circle, hold down cursor and drag.
5. Enter the known concentration of this standard under “Quantity.”
6. Repeat step 16 for the remaining BSA standards and sample spots. Under “Spots” on the top task bar, select all same size spots.
7. Make background corrections by selecting “Background correction” under the “Spots” tab and scroll down to “Manual” in the drop box. *Do not click on “ok” until all background correction boxes are traced near spots. Do not get any portion of the actual spot into the background correction boxes.* Background correction should be conducted for all standards and sample spots.
8. Data, linear regression, and unknown sample quantities (μg) are generated automatically, which can be exported into a Microsoft Excel® or Word document®.

Note: Alternative programs to calculate density of protein spots are Adobe Photoshop® and NIH Image J for Mac or Windows operating systems. NIH Image J can be downloaded free at <http://rsb.info.nih.gov/nih-image/>(Rasband 2011). A short procedure to determine protein concentration from a spot blot using Adobe Photoshop CS4 Extended, version 11.0.2 is detailed below; newer versions may require slight modifications.

Procedure: *An alternative to Imaging System analysis software (e.g., Adobe Photoshop CS4 Extended, version 11.0.2).*

1. Open TIFF (Tagged Image File Format) in photoshop and invert the image from the top task bar under image and adjustments. This changes black to zero.
2. Select Histogram under Window menu on the top task bar and a new dialogue box for histogram analysis should appear. Select expanded view and statistics in the uppermost

right hand corner of the box depicted by an icon with a downward arrow and a series of horizontal lines. When you click on these options using the mouse, a check mark should appear next to both options.

3. Press the shift key plus “M” until a cross cursor appears on the image and a dashed elliptical appears on the tool bar. The tool bar displays many icons of different tools for modifying the image. These icons may be selected by using a mouse instead of using the keyboard shortcut mentioned above.
4. Place cursor over desired spot (e.g., standard spot), click on the spot and drag until circle is formed within the boundary of the spot. If the circle is not correctly placed, make sure the cursor is in the center of the traced circle (cursor changes to a whiteout arrow with a dashed box), click and move until it is within the boundary of the spot.
5. Record mean pixel average (histogram dialogue box on the right of the screen) and move onto the next spot by placing cursor over the traced circle until the whiteout arrow with dashed box appears. Click and drag the traced circle to the next spot and release.
6. Repeat step 5 for all standard spots and sample spots.
7. Next, move the trace circle to areas around all the standard spots and record the mean pixel average. This will be the background subtraction. Do not subtract a background value from an area that is not near the desired spot. All background subtraction must be done for each spot.
8. Enter recorded values into a graphing program (e.g., Microsoft Excel, SigmaPlot®) and generate a standard curve. **See note below.**

Note: A linear regression is used to determine total soluble protein ($\mu\text{g}/\mu\text{L}$) using the average mean pixels (density) of the standard spots for each triplicate. This is plotted in a scattered x/y graph with the known concentrations of BSA standards as the x variable and the average mean pixels as the dependent y variable. A linear trend-line is generated through the data points and interpolation using the formula $y = mx + b$ (slope intercept) is used to calculate the concentrations of unknown samples (Sokal & Rohlf 1995).

Troubleshooting and Optimization

1. When pipetting standards and sample extracts onto filter paper for protein quantification, care must be taken not to press the filter paper with the micropipettor. This creates a depressed mark within the protein spot, which is unstainable and consequently affects protein quantification (Fig. 12a).

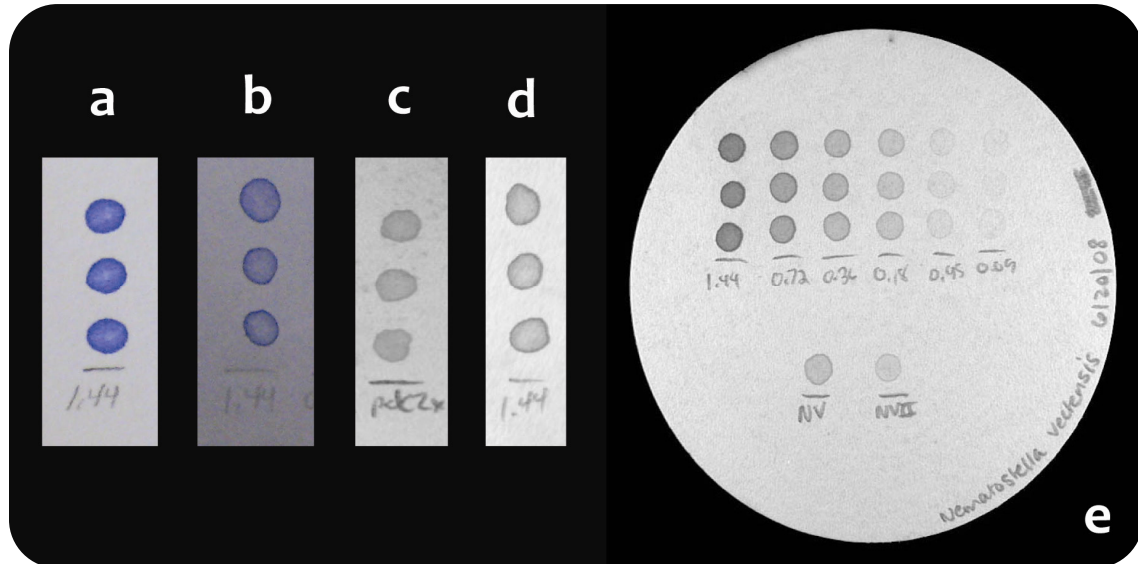


Figure 12. Spot Blot Troubleshooting.

2. A pre-rinse with dH₂O is required before destaining. This step reduces the amount of time it takes to destain, getting rid of the concentrated stain left on the blot. Using destain to do a pre-rinse appears to fix the leftover stain and destaining time can double. See Fig. 12b.
3. Protein quantification of the spot blot takes into account total soluble protein per microliter. Do not exceed the 1 μ L per spot; remove the residual extract on the outside of the micropipette tip. This can be done by dragging the tip against the inside wall of the sample tube away from any liquid.
4. Coomassie stain particulates will create artifacts as shown in Fig. 12c. Filter stain through Whatman No. 5 filter paper to remove any residual dye particulates.
5. Destaining should continue until the background of the filter paper is near white without fading the protein spots. See Fig. 12d.
6. If the standards on the spot blot do not show a two-fold gradation in color from one spot to another, the causes may be: a) standard dilutions were prepared incorrectly, b) the standards were not heated prior to pipetting onto the filter paper, c) spot blot staining time was insufficient, d) Coomassie stain was prepared incorrectly, e) excess destaining occurred, or f) something was bound to the filter paper that interfered with protein staining and binding. A good representative blot of standards is shown in Fig. 12e.
7. A defined area for each 1 μ L spot should be limited within the boundaries of each spot. The defined area of each spot should not include the background (white area outside the stained spot). Background is accounted for separately. If the background is inadvertently included when the defined area of the protein spot is selected (i.e., the circle is not exactly within the stained area), this will result in a reduced pixel value for the spot, the standard curve will not be linear, and the protein concentrations will be inaccurate. See Fig. 13 for a good spot blot and linear curve for *Acropora palmata* sample extracts.

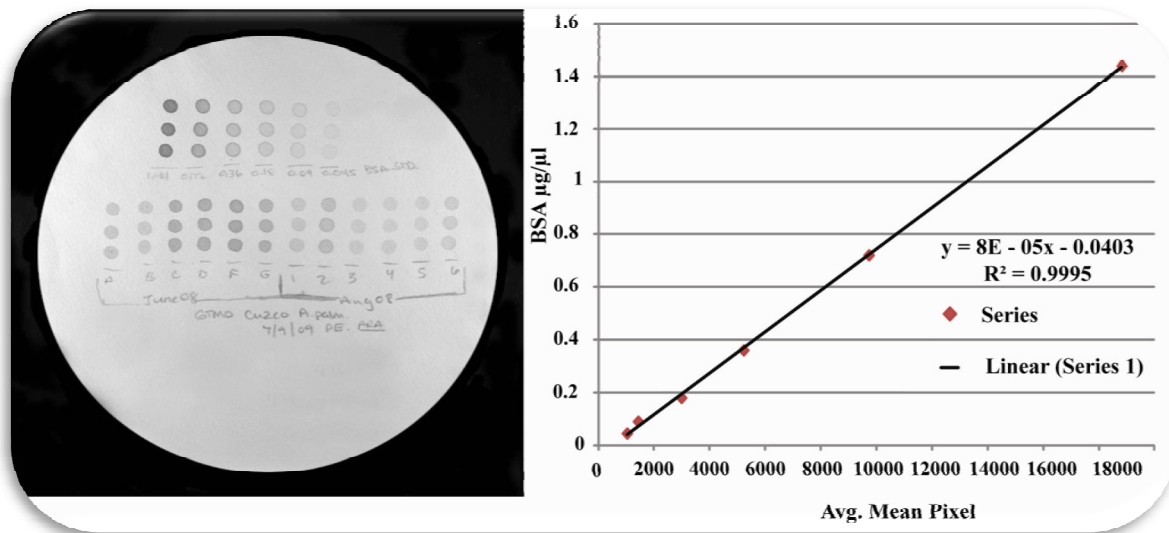


Figure 13. Spot Blot and Linear Curve of Protein Extracts. *Acropora palmata* protein extracts and standard linear curve following spot densitometry are shown here. The degree of confidence with a spot blot will show in the R^2 value of the linear regression.

Time Considerations

Creating a spot blot for protein quantification following Downs (2005) modification of the Ghosh et al. (1988) method can take from 1 hr and 15 min to 2hr for 20 samples. While waiting for the BSA standards to heat to 65°C for 5min, the samples can be spotted on the filter paper leaving an area free for the standards.

Using the digital imager and analytical software from Syngene can take from 15 to 30 min for 20 samples. Using a scanner or camera to capture an image of the spot blot, converting it to a black and white image, and then using a program like Adobe Photoshop to find the mean pixel values can take 30 min or more depending on the user's knowledge of the software.

Protocol 4: Porphyrin Microplate Fluorescence

Protocol 4: Porphyrin Microplate Fluorescence

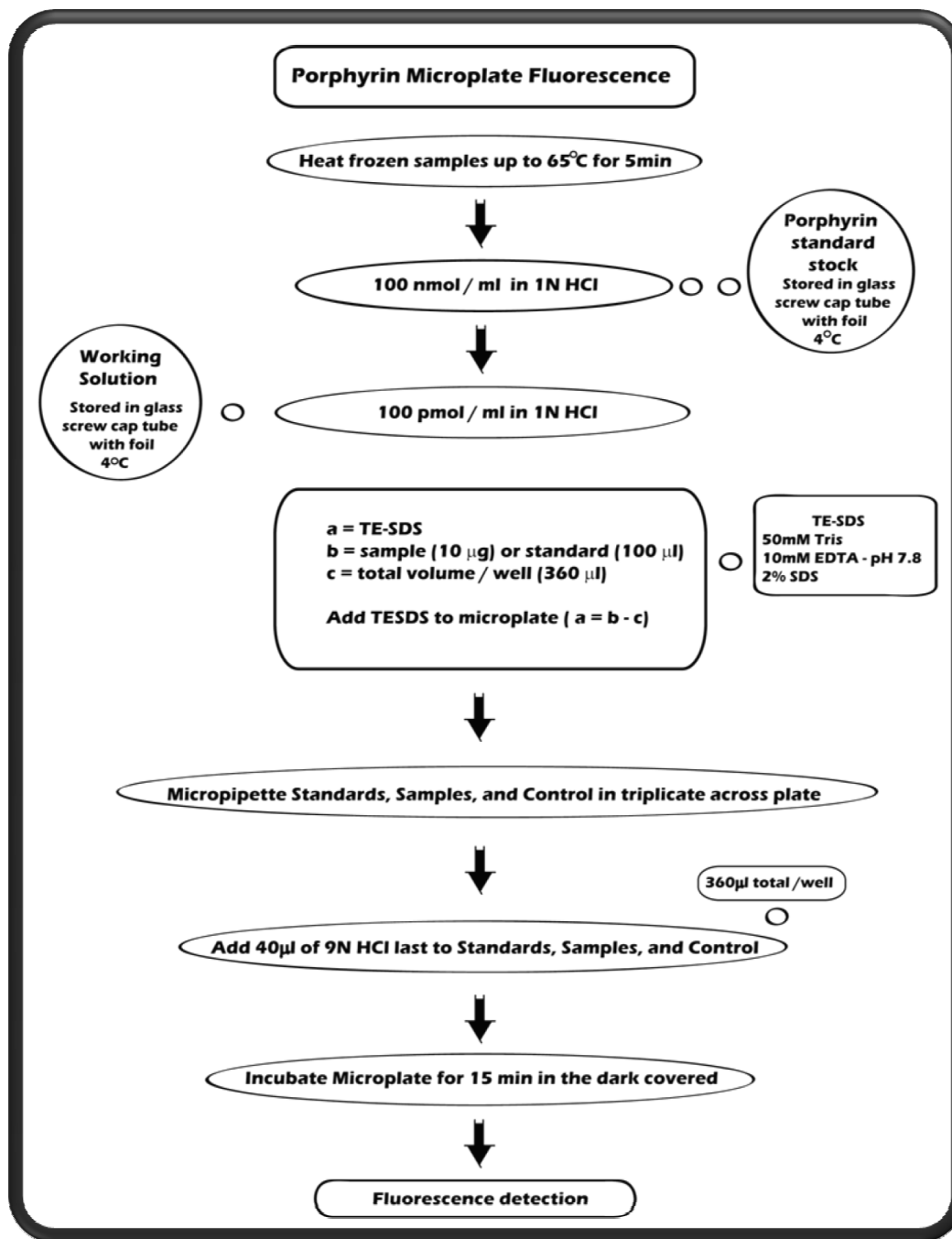


Figure 14. Porphyrin Microplate Fluorescence

A. Porphyrin Detection

Equipment & Supplies Checklist

- 1.5 mL amber microcentrifuge tubes
- 12 N HCl
- 15 mL glass vials with screw on cap wrapped in aluminum foil
- 96 well optical bottom black plate & cover
- Filter defined spectrofluorometer and analysis software
- Micropipettors with 10-100 μL range (P100), 20 – 200 μL range (P200) and 100 – 1000 μL range (P1000)
- Milligram balance
- Minicentrifuge (6 x 1.5 ml rotor with 0.5 ml adaptors), 6000 rpm and 2000 x g
- P200 and P1000 tips
- Refrigerator for storage from 2 to 4°C
- Scanning spectrofluorometer and analysis software
- Statistical software- e.g., Microsoft Excel® or SigmaPlot®
- Timer
- Vortex mixer
- Water bath set to 65°C – minimum capacity of 12 microcentrifuge tubes

Reagent List & Preparation

9 N HCl

Denatured Protein Extracts

Porphyrin Standard Stock 1

- Uroporphyrin III (URO III): 1.807 mg/1 mL of 6N HCl
- Coproporphyrin III (CP III): 1.455 mg/1mL of 6N HCl
- Protoporphyrin IX (PP IX): 1.125 mg/1mL of 12N HCl

Porphyrin Standard Stock 2

- 100 nmol/mL of URO III, CP III, and or PP IX

Porphyrin Standard Working Solution

- 100 pmol/mL of URO III, CP III, and or PP IX

TE-SDS

- 50 mM Tris
- 10 mM EDTA (pH is adjusted to 7.8 with HCl after EDTA is mixed with Tris)
- 2% SDS (add after pH is adjusted to 7.8)

Prepare porphyrin standard stock solution in an amber glass vial or clear glass wrapped with foil. Weigh porphyrin standard with a microgram balance and place into 1.5 mL amber microcentrifuge tube or glass vial. **See above for the different porphyrin formulations.** Reconstitute porphyrin with the appropriate HCl concentration (see above) to 2 $\mu\text{mol}/1\text{ mL}$ and vortex for 1 min. Next, prepare 100 nmol/mL stock solution of the porphyrin standard while

adjusting the final concentration of HCl to 1N. For example: dissolve 1.125 mg of PPIX in 1 mL of 12N HCl. Next, dilute the 2 $\mu\text{mol/mL}$ PP IX with 11 mL of dH_2O , and 8 mL of 1N HCl to a final volume of 20 mL and final concentration of 100 nmol / mL in 1N HCl. Make a working solution from the 100 nmol/mL stock solution by diluting 1:1000 with 1N HCl for a final concentration of 100 pmol/mL. Standards are stored at 4°C, for no longer than 6 months, as per manufacturer's recommendation.

Notes:

1. Porphyrins are light sensitive and consequently this assay must be conducted in a dark room with only indirect light to prevent photo-degradation of standards and samples to be analyzed for porphyrin. Use amber or foil wrapped glass vials to store porphyrin solutions to prevent photo-oxidation.
2. Always warm standards to room temperature and vortex before use. Porphyrin may come out of solution after being stored for a long period in the cold. Heat porphyrin standards to 65°C for 30-60 s and vortex until porphyrin goes back into solution.
3. Acidification is used in this assay to oxidize porphyrinogens to porphyrins for optimal fluorescence detection. It is well known that the spectra of porphyrins are influenced by pH. This step alters the colorless porphyrin precursor to its fluorescent oxidized form, which is the basis for this analysis. Porphyrins in an acidic environment undergo protonation of their pyrrolic nitrogen atoms, thereby enhancing their absorptivity and fluorescence intensity (Rimington 1960; Zhang *et al.* 2005). The Soret band (400-420 nm), used for spectrofluorometric detection and quantification, is optimal in acid solution (Rimington 1960).
4. Black (96-well optical bottom) plates are used in this assay because they absorb light and reduce background scattered light (i.e., background noise) which is imperative for a fluorescence based assay. Luminescence assays use white plates that reflect light and amplify the signal output.

Procedure:

Porphyrin Detection with a Filter Defined Spectrofluorometer (e.g., Biotek Synergy HT)

1. Prepare porphyrin standard stocks 1 & 2, TE-SDS (TE-SDS is leftover from Protocol 3), and 9 N HCl
2. Set up a protocol for the microplate reader (e.g., KC4 software).
 - a. Fluorescence
 - b. 400 ± 15 nm bandwidth excitation filter
 - c. 600 ± 20 nm bandwidth emission filter
 - d. Pre-read blank plate
 - e. Read wells A1-H12
 - f. Photomultiplier (PMT) sensitivity should be optimized for the instrument and assay samples.
3. Define the plate layout with standards, samples, and assay controls.

4. Read blank plate. This must be done before loading standards and samples onto plate.
5. Prepare a set of porphyrin standards in 1.5 mL amber tubes: 5000, 3500, 3000, 2500, 2000, 1000 and 500 fmol in a standard volume of 100 μ L for each (e.g., 5000 fmol/100 μ L; 3500 fmol/100 μ L) from standard stock 2 and TE-SDS as diluent.
6. Thaw frozen sample extracts to 65°C for 5 min, vortex, and spin (2000 x *g*) using the minicentrifuge. Dilute samples to a final concentration of 10 μ g TSP with TE-SDS in 1.5 mL amber tubes for a total volume of 320 μ L.
7. Plate 320 μ L of extraction buffer, standard and samples per well in triplicate as shown in the plate layout below (Fig. 15).
8. Add 40 μ L of 9N hydrochloric acid to each well to bring the final volume to 360 μ L. Cover the plate and incubate at room temperature for 15 min prior to reading the prepared plate on the spectrofluorometer.
9. Read plate, save data, and export raw data into spreadsheet (refer to manufacturer’s manual for detailed guidance on the software).

	1	2	3	4	5	6	7	8	9	10	11	12
A	EB	s1	s1	s1	2	s9	s9	s9	5	s17	s17	s17
B	EB	s2	s2	s2	3	s10	s10	s10	5	s18	s18	s18
C	EB	s3	s3	s3	3	s11	s11	s11	6	s19	s19	s19
D	1	s4	s4	s4	3	s12	s12	s12	6	s20	s20	s20
E	1	s5	s5	s5	4	s13	s13	s13	6	s21	s21	s21
F	1	s6	s6	s6	4	s14	s14	s14	7	s22	s22	s22
G	2	s7	s7	s7	4	s15	s15	s15	7	s23	s23	s23
H	2	s8	s8	s8	5	s16	s16	s16	7	s24	s24	s24

Figure 15. Porphyrin Microplate Layout. Numbers 1-7 highlighted in blue represent the porphyrin standards from 500 fmol to 5,000 fmol. Samples are designated here as s1-s25 and the assay control as “EB” for extraction buffer.

Procedure:

Porphyrin detection with a Scanning Spectrofluorometer (e.g., SPECTRAmax®)

1. On SPECTRAmax PRO® (software) set up the parameters for the assay.
 - a. Fix excitation to 410 nm to scan emission or use a fixed emission of 650 nm to scan excitation under Spectrum.
 - b. Enter the range the sample is expected to emit (λ 500-750 nm) and include the number of Steps (increments between wavelengths, i. e., Step 3).
 - c. PMT (Photomultiplier sensitivity): Medium (recommended)
 - d. Reads/well: 3
 - e. Auto calibrate ON
 - f. Strips: 1-9 (select how many columns and rows for reading)
 - g. Auto Read OFF
 - h. Speed Read OFF (less accurate if on)
 - i. Template: Highlight wells and assign accordingly (Rows A-H; Columns 1-12).
 - i. Blanks

- ii. Standards
 - iii. Unknowns
 - j. Reduction: read lambda at maximum 0-20000 rfu
2. Load microplate onto reader after the instrument has auto-calibrated, once powered on. Auto-calibration should not be skipped or terminated.
 3. Export raw data into text file.

B. Porphyrin Quantification

Procedure:

1. Export raw data into an Excel spreadsheet or text file that can be transferred later to graphing software. There may be accompanying software with the instrument that enables automatic generation of linear regression (e.g., KC4 software).
2. Select the data set (assorted by PMT sensitivity) that has the highest relative fluorescence units (RFUs) for the porphyrin standards without reaching saturation. Saturation may be denoted by some character like an exclamation mark.
3. Determine the average of each triplicate of standards and unknown samples. Graph the standards and determine the formula for the standard curve. Evaluate the R^2 values of the linear regression to determine the suitability of the curve.
4. Use slope-intercept formula to determine porphyrin concentration for each sample.

Notes:

1. Extraction buffer and hydrochloric acid treatment used to acid hydrolyze samples do not show a significant interference in the detected Uroporphyrin III Standard. Individual components of the buffer were evaluated with respect to fluorescence (4% PPV, 0.5% DMSO, 2% SDS, 10 mM DTT, and 0.05 mM DM) and interference with porphyrin detection (Fig. 16a). It was determined that the denaturing buffer used for soluble protein fractions from corals in western blots does not interfere with the detection of porphyrin within a sample.
2. Protoporphyrin IX was chosen as the standard for this assay after evaluating all three choices: URO III, CP III, and PP IX. This standard was chosen based on three criteria: 1) offers the greatest dynamic range within the narrow diagnostic wavelengths (Ex: 410 nm, Em: 600 nm), 2) wavelengths that do not interfere with chlorophyll emission from the symbiotic algae in coral tissues, and 3) the most cost-effective for a routine diagnostic assay (Fig. 16b).
3. Initially, a 400 ± 17.5 nm-bandwidth excitation filter and 645 ± 20 nm emission filter was used with the spectrofluorometer. These filters were shown to detect total porphyrins in a microplate fluorescence assay in chicken embryo hepatocyte cultures dosed with polychlorinated biphenyls (PCBs), and reported to cause porphyrin accumulation in humans and animals (Kennedy *et al.* 1995). However, this emission filter could not be used for samples that contained chlorin (e.g., chlorophylls). Using the scanning

spectrofluorometer, it was determined that the spectra of chlorophyll *a* overlapped with the three diagnostic porphyrins (Uroporphyrin III, Coproporphyrin III, and Protoporphyrin IX) from 630 to 730 nm when excited at 410 nm (Fig. 16c). As a result, an alternative emission filter, 600 ± 20 nm, was used to minimize chlorophyll interference (Fig. 16d).

4. Solubility of porphyrins is determined by the number of carboxylate groups (Perkins *et al.* 1989; Deacon 2008). Uroporphyrin (8 carboxylate groups) is the most soluble porphyrin within an aqueous solution, coproporphyrin (4 carboxylate groups) is intermediately soluble and protoporphyrin (2 carboxylate groups) is the least soluble. Protoporphyrin IX solubility with hydrochloric acid was determined by reconstitution in 6N HCl (1mg/mL), 9N HCl and finally 12N HCl, vortexed for 1 min, low speed centrifugation, vortexed for 30 s and diluted to 1N HCl. Undissolved protoporphyrin was observed after low speed centrifugation of the solution for both lower HCl concentrations. No particulates were observed in the concentrated HCl solution. Uroporphyrin III and coproporphyrin III were completely solubilized in 6N HCl and also remained in solution when diluted to 1N HCl. For stability and long-term storage (6 month maximum) at 4°C, glass capped vials wrapped with aluminum foil were used to prevent photo-degradation.
5. High grade, fresh chemicals are recommended for this assay along with denaturing buffer that should be made fresh on the day of protein extraction to achieve optimal results. As mentioned above in the protein extraction protocol, protease inhibitors also have a short half-life in the denaturing buffer.
6. Frontier Scientific, the supplier of porphyrin products, recommends reconstituted porphyrin in 1N HCl storage for up to 6 months. To prevent photo-degradation of the porphyrin standards, use amber or foil-wrapped glassware and work in a dark room with incident light or a room with red light. Make sure porphyrin standards are contained properly, using amber microcentrifuge tubes for standards and samples for dilutions made before dispensing into a microplate.

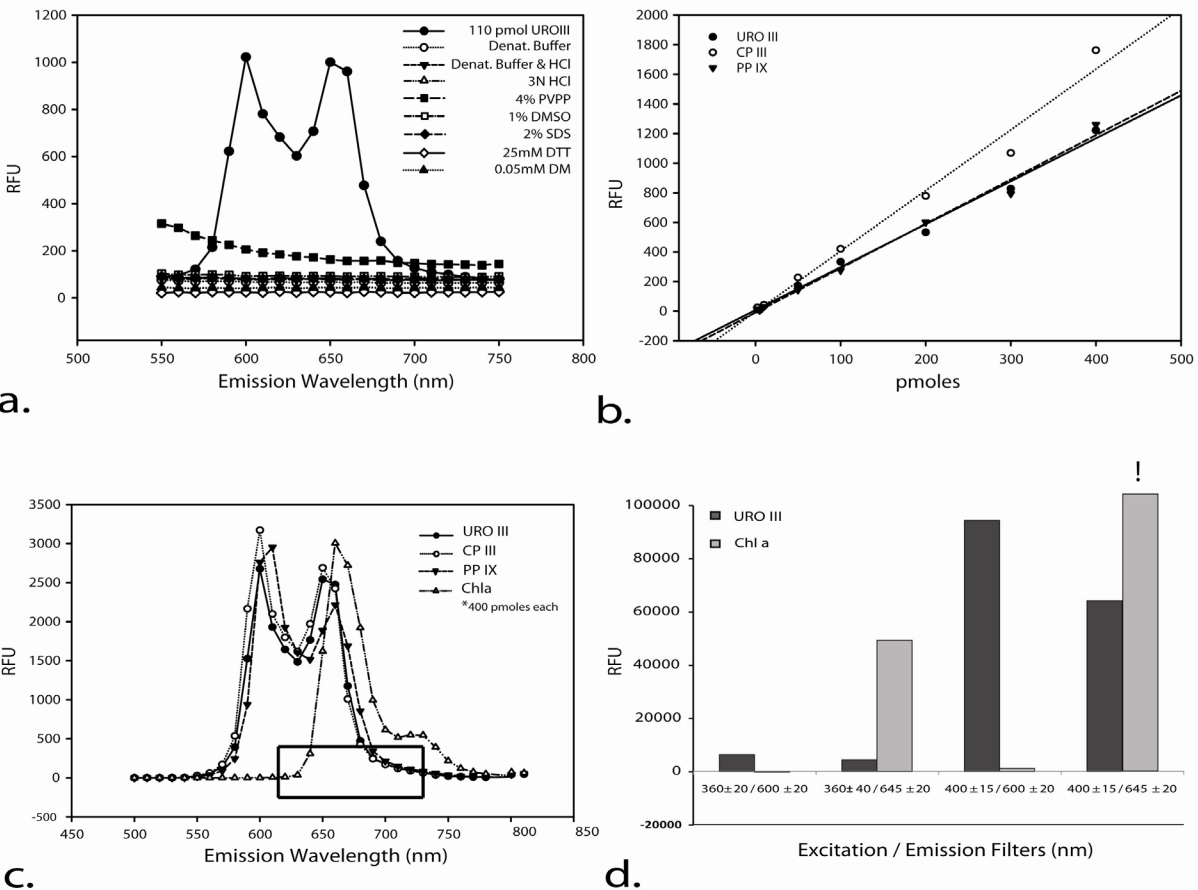


Figure 16. Porphyrin Assay Optimization and Validation. (a.) **Denaturing Buffer Validation.** The emission spectrum of URO III and individual buffer components HCl, PVPP, DMSO, SDS, DTT, and DM was scanned from 500-800 nm with excitation fixed at 410 nm. Polyvinylpyrrolidone, showed low-level background fluorescence that interferes with porphyrin detection if it is not removed during centrifugation in the extraction procedure, all other components showed no significant interference. (b.) **Selection of a Porphyrin Standard.** The linear relationship of URO III, CP III, and PP IX was examined from an 8 point calibrant curve (400 pmol – 2 pmol). Protoporphyrin IX was chosen for optimal linearity from the lowest to the highest values on the standard curve and it was the most cost effective of those examined. (c) **Porphyrin and Chlorophyll Interference.** Uroporphyrin III, CP III, and PP IX detection was examined for interference by chlorophyll *a*. The emission spectrum was scanned from 450-850 nm with excitation fixed at 410 nm. Chlorophyll *a* interferes with porphyrin fluorescence from 640-780nm. (d) **Spectrofluorometric Filter Validation.** Four excitation/emission filter combinations were examined, based on the porphyrin and chlorophyll *a* interference shown in (c) to optimize porphyrin signal and reduce/eliminate chlorophyll interference. Uroporphyrin III fluorescence was maximized and chlorophyll *a* minimized without significant interference using a filter with excitation maximum of 400 ± 15 nm and emission maximum of 600 ± 20 nm. All other filter sets show that chlorophyll *a* is either saturated (shown by exclamation mark) or URO III fluorescence is significantly reduced.

Time Considerations

Porphyrin standard preparation can take up to 30 min. It takes 25 min (25 samples) for standard and sample dilutions with appropriate amounts of TE-SDS, once samples are thawed for 5 min in a 65°C water bath. Plating, incubation with HCl, and reading the microplate can take up to 45 min.

Plating Considerations

The TE-SDS buffer has a high concentration of SDS which can create a lot of bubbles in the wells that interfere with detection by quenching fluorescence. To prevent bubbles from forming, draw pipette tip up along the side of the well while dispensing liquid and do not expel past the final stop of the micropipette plunger.

Case Study

A study was conducted using 10 different species of Cnidaria from different habitats to provide an example of the application of this method and the range of total porphyrin levels that a user may encounter (Table 2). See Fig. 17 for examples of six species used in this study. Field collected (F) and wild specimens cultured under laboratory conditions (LC) at least 6 months were used in this study. The freezer mill was used to homogenize all specimens except *Aiptasia pulchella* and *Nematostella vectensis*. These two species were prepared using the soft tissue homogenization procedure for non-scleractinian cnidarians (pg. 10). Care was taken to remove algae from the coral fragments before grinding. The only specimen with visible endolithic algae was from *Montastrea annularis*, which had been cultured in the laboratory for over 10 years.

Tissue Processing

The stony coral specimens were ground using the Freezer mill 6850. A tissue homogenate with the consistency of a fine powder was achieved with a 3 min (T1) grinding cycle and impact frequency (rate) of 10 cycles per sec. The freezer mill requires a 90 min pre-cooling period (T3) with liquid nitrogen before processing samples. A cooling period (T2) in between grinding samples is necessary to chill grinding accessories and sample within the freezer mill before grinding commencement. This option was replaced by chilling samples and grinding accessories in separate insulated containers with LN₂. While one grinding cycle is running, chilling vials, end plugs and samples will reduce processing time. Specimens with soft tissue (anemone and soft coral) were homogenized manually using a micro-pestle homogenizer that fits into a microcentrifuge tube. Tissue extracts were prepared according to the protocols outlined in this document.

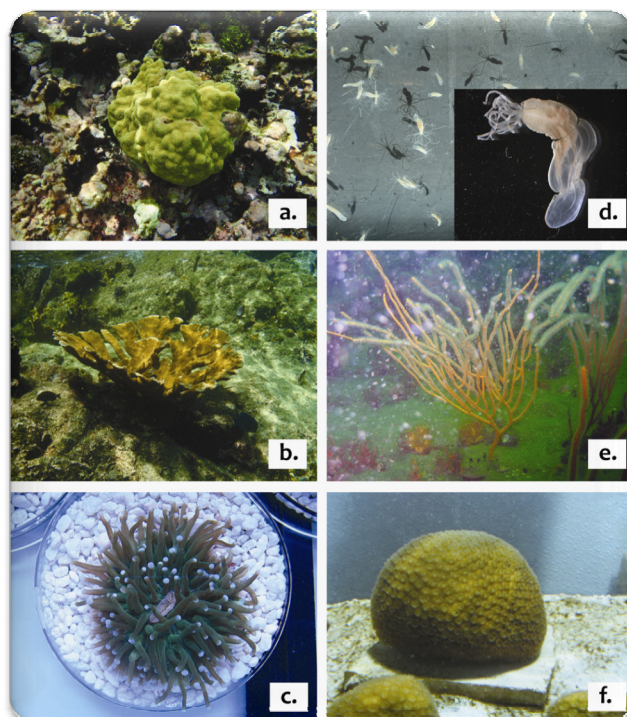


Figure 17. Examples of Cnidarians used in this Study. Scleractinian corals are shown in panels a) *Porites astreoides* in the U.S. Virgin Islands, b) *Acropora palmata* in the U.S. Virgin Islands, c) *Heliofungia* sp. from the Indo-pacific, and f) *Montastraea annularis* cultured in the laboratory. Other cnidarians used in the case study are shown in panels d) *Nematostella vectensis* from a Charleston, SC tidal marsh and e) *Leptogorgia virgulata* from Grey's Reef, coastal Georgia. Photo credits: Craig Downs (a, b), Athena Avadanei (c, d), Julie Higgins (e), and Tom Bartlett (f).

Results:

In this study, *Aiptasia pulchella* and the soft coral, *Leptogorgia virgulata* (1.2-1.4 µg/µL) had the highest protein concentrations. These species lack a skeleton and generally produce an extract with higher protein yield (see Table 2). Protein concentrations from stony coral species ranged from 0.1 to 1.4 µg/µL. Denaturing buffer to tissue ratios should yield optimal protein concentrations in the range of 0.5 to 1.0 µg / µL. Samples that are too dilute cannot be used in certain applications because of volume constraints (e.g., western blotting or ELISAs). If extracts are too concentrated, it is often the case that the samples will not be denatured properly. This condition cannot be corrected by dilution, since it is the initial ratio of denaturant to tissue and mixing that is responsible for the success or failure of the extraction. The ratio of buffer to tissue as well as the concentration of the denaturants (e.g., SDS, DTT) and other buffer components to reduce mucus effects have to be experimentally determined for each species.

Table 2. Total Soluble Protein Range from Surveyed Cnidarians. * Each *N. vectensis* was weighed on a milligram balance. A whole animal was used per extract. A key for color abbreviations: DY= dark yellow, PY = pale yellow, MY = medium yellow, YB= yellow brown, GDY = green & dark yellow, Bg = beige, PK = pink, and PO = pale orange.

Species	Tissue Volume (µL)	Denaturing Buffer Volume (µL)	Protein extract color	protein range µg/µL
<i>Acropora chesterfieldensis</i>	150	500	PY- MY, YB, GDY	0.36-0.50
<i>Acropora millepora</i>	150	600	PY - MY	0.80-0.84
<i>Acropora palmata</i>	250	700	Bg	0.33-0.86
<i>Aiptasia pulchella</i>	100	500	PY-DY	1.2-1.4
<i>Fungia fungites</i>	200	600	PO, Bg	0.52-0.93
<i>Leptogorgia virgulata</i>	200	600	MO MY, Green foam on	1.2-1.4
<i>Montastrea annularis</i>	150	500	top	0.23-0.50
<i>Nematostella vectensis</i>	*50-170mg	700	PW	0.26-1.4
<i>Porites astreoides</i>	150	500	PY-DY	0.26-1.4
<i>Porites lobata</i>	150	400	PY-DY,PO,PK	0.69-1.4
<i>Porites divaricata</i>	200	800	PY-DY	0.10-0.64

Nominal levels of porphyrin often vary from one species to another; therefore, it is critical to determine the nominal range for each species when optimizing this porphyrin assay. For field studies this can be accomplished by obtaining specimens from well characterized reference sites or specimens that have been raised in contaminant free aquaculture systems.

A wide range of porphyrin levels can be found in the wild as well as in laboratory cultured specimens, as shown in Table 3. The anemone and soft coral appear to have low porphyrin levels compared to the stony coral, ranging from 28.1 – 44.9 fmol/µg, varying with the individual. Stony coral porphyrin levels ranged from 47.7-399.1 fmol/µg, varying with species as well as individual. It should be noted that the detection limit of this assay has been determined

as 10 fmol / μg of TSP with linearity up to 500 fmol. Specimens exposed to toxicants may present with much higher levels, in which case a dilution of samples must be re-analyzed.

Table 3. Total Porphyrin Levels for Select Cnidarians from TSP. Samples of 10 different species from the field (F) or originally from the wild and cultured in the lab (LC) were evaluated for porphyrin levels. Shown here is the total porphyrin in fmol / μg of total soluble protein (TSP).

Species	fmol/ μg	SE	Origin
<i>Aiptasia pulchella</i> (n=3)	86.8	39.0	LC
	194.3		
	211.4		
<i>Montastrea annularis</i> (n=3)	199.2	40.8	LC
	85.1		
	69.8		
<i>Porites divaricata</i> (n=3)	128.4	27.4	LC
	79.7		
	174.7		
<i>Fungia fungites</i> (n=3)	83.7	12.6	LC
	114.5		
	72.1		
<i>Acropora chesterfieldensis</i> (n=3)	94.2	16.1	LC
	47.7		
	97.8		
<i>Acropora millepora</i> (n=2)	118.5	12.7	LC
	144.0		
<i>Nematostella vectensis</i> (n=3)	33.6	2.2	LC
	28.1		
	35.5		
<i>Leptogorgia virgulata</i> (n=3)	44.9	4.4	F
	29.7		
	35.6		
<i>Acropora palmata</i> (n=5)	66.3	12.5	F
	135.0		
	100.5		
	118.5		
	79.8		
<i>Montastraea faveolata</i> (n=3)	309.0	74.2	F
	399.1		
	145.7		

Conclusions:

This document provides the first detailed method encompassed in four interdependent protocols for analyzing cnidarian porphyrin levels in tissues extracted under denaturing conditions for total soluble protein and their detection with a fluorescence microplate reader. We also provide validation evidence showing that the denaturing buffer components do not interfere with fluorescence detection and that the assay has a detection limit of 10 fmol of total porphyrin per microgram of total soluble protein. Finally we demonstrate porphyrin levels can vary across species and among individuals, as well as the concentration ranges that may be encountered when using this method. This is important to recognize when developing any given study design as well as for the proper application of this method as a diagnostic.

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