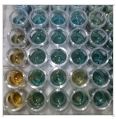

Apr 09, 2020

Microplate Assay for Quantification of Soluble Protein in Ground Coral Samples

Rowan Mclachlan[1], James Price[1], Kerri Dobson[1], Noah Weisleder[1], Andrea Grottoli[1]

[1]The Ohio State University

1 Works for me dx.doi.org/10.17504/protocols.io.bdc8i2zw

Rowan Mclachlan
The Ohio State University

ABSTRACT

This method for extracting protein from ground coral samples is based on the Bradford assay for the colorimetric detection and quantification of total protein (Bradford, 1976) and is compared to a known standard dilution of bovine serum albumin (BSA). Pierce Inc. and Bio-Rad have developed the reagents and standards necessary for completing the extraction. There are five parts to quantifying total soluble protein in ground corals: 1) grind and sub-sample the coral and store at -80 °C until ready to extract, 2) solubilize protein via cell disruption [detergent lysis and freeze-thaw lysis], 3) separate the dissolved protein from tissue and skeletal particles, 4) quantify the protein concentration via Bradford microassay procedure, and 5) standardize the protein concentration to ash-free dry weight (AFDW).

This method was originally developed by Rowan McLachlan with the assistance of Jamie Price and Kerri Dobson and with the guidance of Dr. Noah Weisleder and Andréa Grottoli at The Ohio State University. This protocol was written by Rowan McLachlan and reviewed by all co-authors.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

MATERIALS TEXT

LEGEND

[1] for Grinding of Coral Fragments

[2] for Solubilizing Protein

[3] for Separating Dissolved Protein from Tissue and Skeletal Particles

[4] for Bradford Microassay Procedure

[5] for AFDW Procedure

Reusable materials:

Citation: Rowan Mclachlan, James Price, Kerri Dobson, Noah Weisleder, Andrea Grottoli (04/09/2020). Microplate Assay for Quantification of Soluble Protein in Ground Coral Samples. https://dx.doi.org/10.17504/protocols.io.bdc8i2zw

- Mortar and pestles [1]
- Plastic spatula [1]
- Metal spatula [1]
- Glass graduated cylinder [2,4]
- 200 ml Nalgene bottle for storing RIPA buffer [2]
- 1000 µl capacity pipette [2,4]
- 1L capacity dewer for liquid nitrogen
- Test tube racks [2]
- Water-resistant marker pen [4]
- 100 ml glass beaker [4]
- Erlenmeyer flask [4]
- 20 µl capacity pipette [4]
- 20 µl capacity multi-channel pipette [2]
- 200 µl capacity multi-channel pipette [4]

Disposable materials:

- Aluminum weighing pans [1]
- 15 ml polypropylene centrifuge tubes [1]
- 50 ml polypropylene centrifuge tubes [1]
- 200–1000 µl pipette tips [2]
- Kim wipes [3]
- Ice bucket [3]
- Disposable plastic transfer pipettes [3]
- 2 ml conical Eppendorf™ tubes [3]
- 5 ml conical Eppendorf™ tubes [4]
- Parafilm [4]
- Flat-bottom medium binding clear 96-Well polystyrene microplates (must be suitable for spectrophotometry) [4]
- 2–20 µl pipette tips [4]
- Disposable pipetting reservoirs [4]
- 20–200 µl pipette tips [4]

Equipment:

- -80 °C freezer [1,2,3]
- Weighing balance accurate to 4 decimal places [1]
- Vortex [2]
- Refrigerated swinging bucket centrifuge [3]
- 96-well plate shaker [4]
- Benchtop Multi-Mode Microplate reader [4]
- Drying oven [5]
- Muffle furnace [5]

Chemicals:

- Ultrapure water [2,4]
- 15 ml concentrated (10X) Radioimmunoprecipitation (RIPA) buffer [2]
- 500 ml liquid nitrogen [2]
- Crushed ice [3]
- Bovine Serum Albumin (BSA) 2 mg/mL standard ampules [4]
- 450 ml Bio-Rad protein assay dye reagent concentrate [4]

Software:

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- SoftmaxPro 5.4.1 (or more recent version) [4]
- Microsoft Excel [5]

SAFETY WARNINGS

Lab Safety

This procedure uses hazardous chemicals:

- Complete your institutions chemical safety training before working with liquid nitrogen.
- Read the manufacturer's instructions for the Bio-Rad protein assay.
- Read the MSDS forms for each chemical in the procedure.
- Use powder-free nitrile exam gloves throughout the procedure.
- Wear a lab coat and safety glasses throughout the procedure.
- Dispose of all chemical waste in appropriately labeled containers.

Preparation of ground coral samples

1 **Grind coral fragments.** Grind frozen coral fragments into a homogeneous paste using a chilled mortar and pestle, or within an ice-bath. Mortar and pestle can be chilled using liquid nitrogen or by placing in -80 °C freezer ~20 minutes prior to use. Grind the paste into as fine a consistency as possible to get evenly fine skeletal grains.

2 **Weigh and partition ground coral paste.** Place a pre-baked aluminum weighing pan on the balance and tare (i.e. zero). Remove the pan, and using a rubber spatula, transfer all the ground coral material from the mortar into the pan. Place the pan on the balance and record the *total wet weight of the ground coral fragment (grams).* Using a metal spatula, measure ~ 0.5 g of wet paste from the pan into a pre-labeled 15 ml polypropylene centrifuge tube. Record the exact weight of the paste removed as the *wet weight of subsample*. Return the 15 ml tube to the -80 °C freezer as soon as possible for storage until you are ready to go to the next step. The remaining ground coral material in the pan not allocated for protein analysis can be transferred to a separate 50 ml centrifuge tube and archived in a -80 °C freezer for other future analyses.

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Solubilize protein via cell distruption

3 Detergent lysis buffer and freeze-thaw lysis are two methods of cell disruption used on the samples to ensure that all of the animal and Symbiodiniaceae cells are lysed, allowing protein to be extracted from the samples and solubilized into the buffer.

Detergent lysis. Prepare a 1X solution of Radioimmunoprecipitation (RIPA) lysis buffer. Using a glass graduated cylinder, add 15 ml of concentrated 10X buffer into a 200 ml Nalgene bottle. Next, measure 135 ml of ultrapure water, and add to the same Nalgene bottle. Gently swirl the diluted buffer solution until thoroughly mixed.

Remove samples (~0.5 g of ground material in 15 ml centrifuge tube from step 2) from the -80 °C freezer, and allow to partially defrost at room temperature for 2–5 mins (Fig. 1). Using a 100-1000 µl capacity pipette and one clean tip per sample, add 1000 µL of diluted 1X RIPA buffer to each sample centrifuge tube. Using a clean metal spatula, scrape the inner walls of each centrifuge tube to ensure that all particulate matter is submerged in the buffer at the base of the tube. Place samples in a bucket of crushed ice to keep cool (Fig. 1). When RIPA has been added to all tubes, vigorously vortex each sample for 30 seconds.

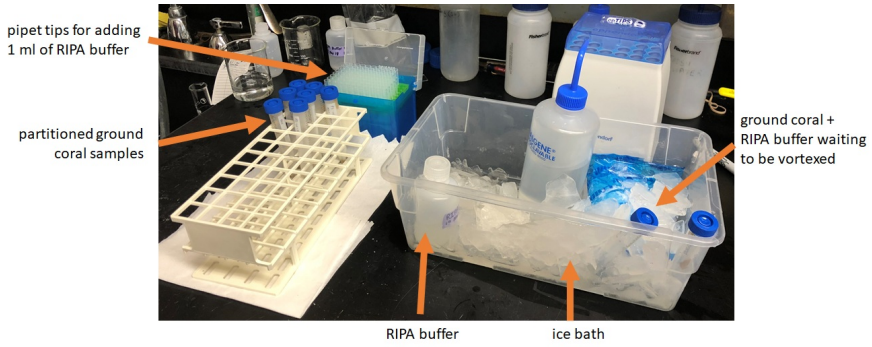

Fig. 1. Setup for adding RIPA buffer to ground coral samples.

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4 **Three cycles of freeze-thaw lysis.**
Freeze #1: Partially submerge each centrifuge tube in a small dewar of liquid nitrogen (maximum depth 5 cm) (Fig. 2) for ~10 seconds or until the sample has frozen completely. *Ensure that you do not submerge the entire 15 ml centrifuge tube in the liquid nitrogen as it poses an EXPLOSION RISK.* Transfer each tube to a room temperature water bath (Fig. 2) for ~ 2 minutes, or until the sample has defrosted completely. Vortex each sample for 30 seconds.

Freeze #2: When samples are defrosted, partially submerge them again in the bath of liquid nitrogen for ~10 seconds or until they have frozen completely. Transfer each tube to a room temperature water bath for ~ 2 minutes, or until the samples have defrosted completely. Vortex each sample for 30 seconds.

Freeze #3: After vortexing, return all samples back to the -80 °C freezer. This is considered the third cycle of freeze-thaw lysis. Do not take the samples out of the freezer until you are ready to go to the next step. Alternatively, if you are ready to proceed to the next step, submerge samples again in liquid nitrogen followed by the water bath as before and proceed directly to step 5.

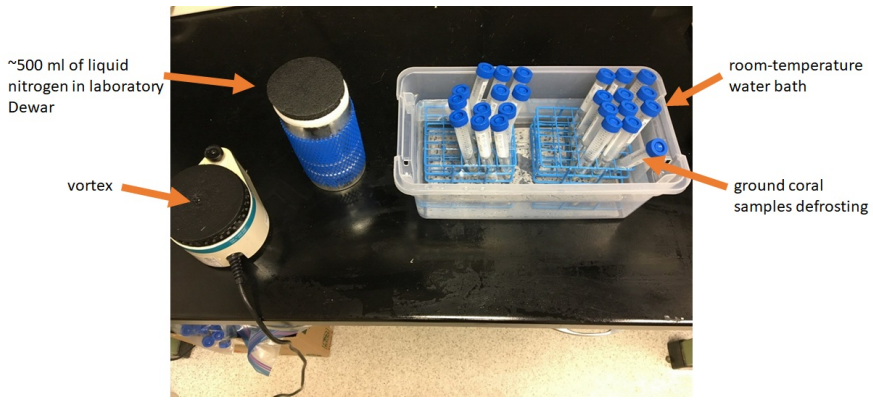

Fig. 2. Setup for three-cycles of freeze-thaw lysis on ground coral samples.

Separation of dissolved and particulate matter

5 **Turn on the centrifuge.** Turn on the swing-bucket centrifuge and set the temperature to 4 °C. It will take ~ 10 mins for the condenser to reach the correct temperature.

Note: that using a non-chilled centrifuge increases the likelihood that protein degradation will occur. It is not recommended. However, if unavoidable, we recommend modifying the centrifuge procedure described in step 7 as follows: 5-minute *centrifuge cycle at 4000 rpm, place samples in an ice bath for 5 minutes, followed by another 5-minute centrifuge cycle at 4000 rpm.*

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6 **Defrost samples.** Remove samples from the -80 °C freezer, and defrost them in a water bath with room-temperature water for ~ 3 minutes (Fig. 3). When defrosted, dry the outside of the centrifuge tubes using a paper towel or Kim-wipes. Vortex each tube for ~30 seconds.

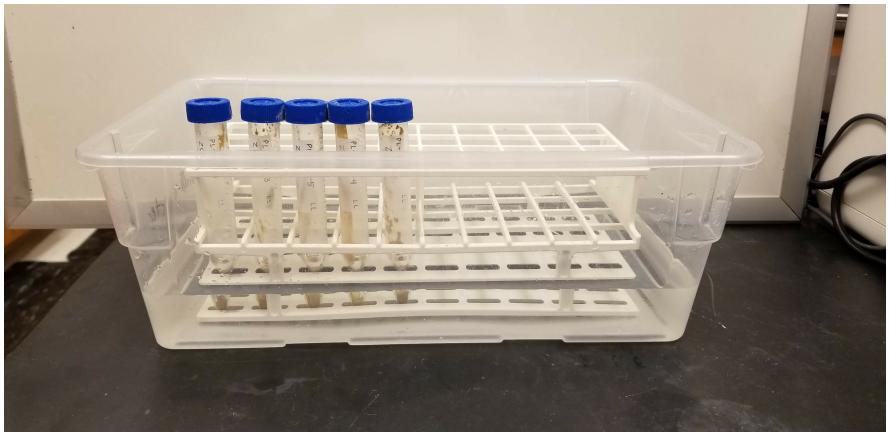

Fig. 3. Water bath for defrosting ground coral samples.

7 **Centrifuge samples.** Load sample tubes into the swing-bucket centrifuge in a balanced configuration (Fig. 4). Centrifuge samples for 20 mins at 4200 rpm / 4122 x g, at 4 °C.

Fig. 4. Example of balanced configuration of 15 ml centrifuge tubes inside a refidgerated swing-bucket centrifuge.

At the end of the spin, gently remove the centrifuge tubes, so as not to re-suspend the particulate matter. Place samples upright in a bucket of crushed ice (Fig. 5).

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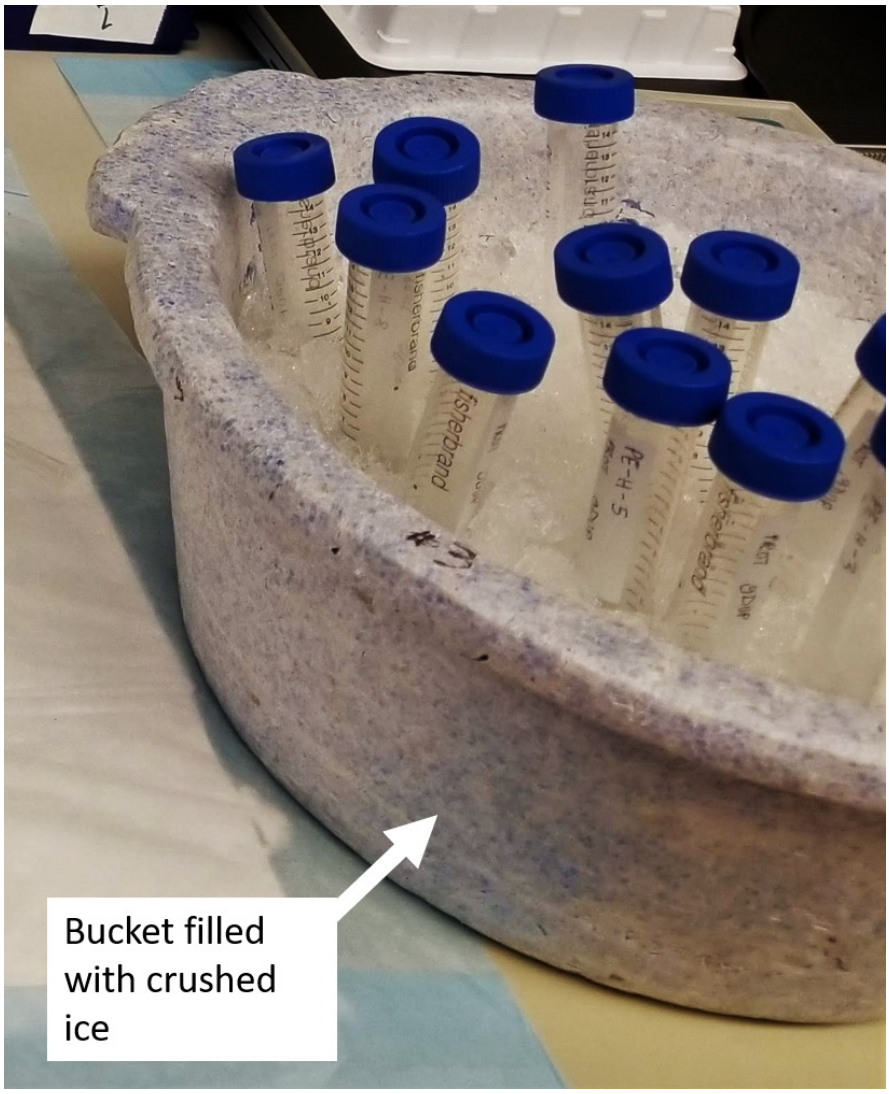

Fig. 5. Ice bucket for storing protein samples.

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8 **Separate middle-phase of supernatant.** Using clean disposable plastic transfer pipettes, remove the supernatant from each 15 ml centrifuge tube, and transfer to a pre-labeled 2 ml Eppendorf tube. Be careful to only collect the middle phase of the supernatant, making sure to avoid sampling the upper phase (whitish matrix) or disturbing the lower phase (particulate matter) (Fig. 6). It is not necessary to collect *all* of the supernatant, and instead it is better to prioritize *not* collecting any particulate matter. While not all samples will have the upper matrix layer, it can be common in species such as corals in the genus *Porites*.

A video of the supernant collection step can be found here: https://youtu.be/XilRFNagf2k

Store the 2 ml Eppendorfs containing the supernatant of solubilized protein upright in a bucket of crushed ice. Return the 15 ml centrifuge tubes containing the white matrix and particulate matter to the -80 °C freezer, as these will be needed later for ash-free dry weight quantification.

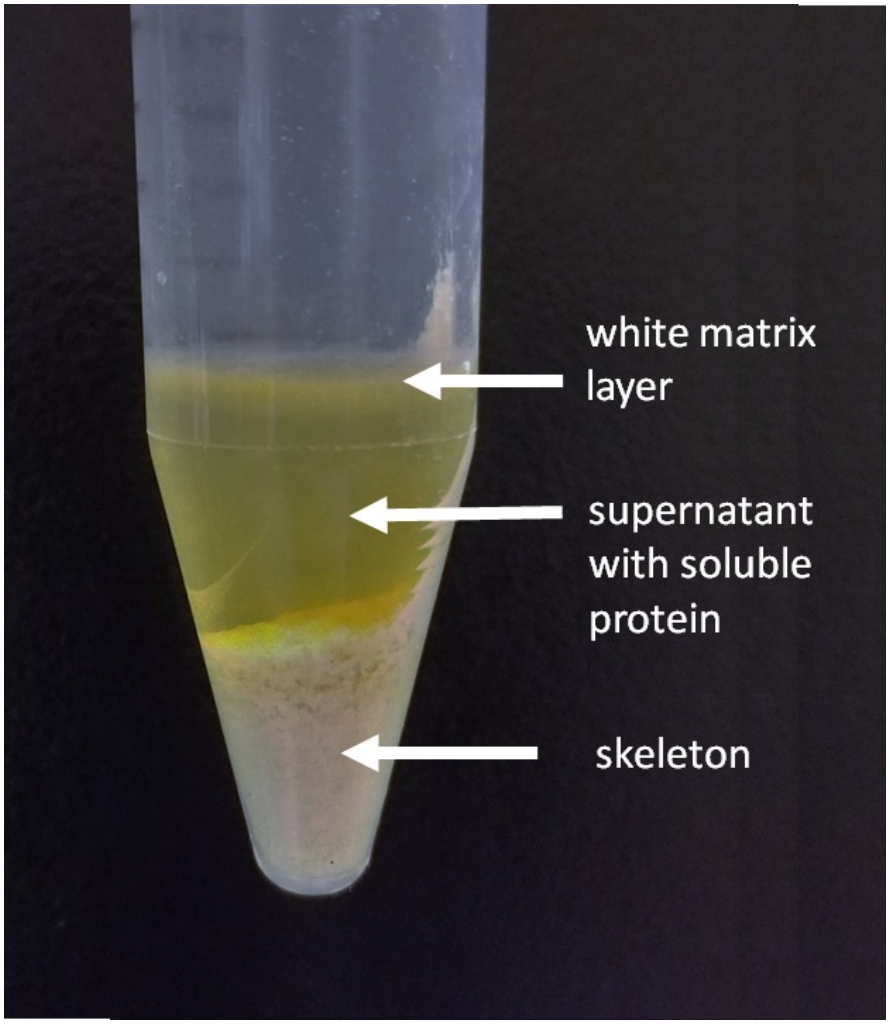

Fig. 6. Diagram illustrating the density layers formed after centrifuging the ground coral + RIPA samples for 20 minutes at 4000 rpm.

Bradford microassay procedure

9 **Prepare BSA standards.** Bovine serum albumin (BSA) standards should be prepared on the same day that the Bradford Assay will be conducted. Accurate pipetting technique is critical for creating a good calibration curve. Use new pipette tips for each dilution and transfer.

Using a water-resistant marker pen, pre-label eight 5 ml conical Eppendorf tubes on the lid and sides with letters A - H (Fig. 7).

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Prepare a series of diluted BSA standards ranging from 0 to 500 µg/ml. Dilute a 1 ml ampule of BSA (= stock) as indicated in Table 1. Use ultrapure water as the diluent. Mix Eppendorfs gently by tipping and swirling the tubes - do not shake tubes as shaking creates micro-bubbles and interferes with accurate volume dispensation. If bubbles arise, try centrifuging the tube for 1 min at 4000 rpm.

Dilution ID#	Volume of Diluent (ultrapure water)	Volume and Source of BSA	Final BSA Conc.
A	1500 µl	500 µl of stock (2000 µg/ml)	500 µg/ml
B	800 µl	200 µl of stock (2000 µg/ml)	400 µg/ml
C	1700 µl	300 µl of stock (2000 µg/ml)	300 µg/ml
D	1500 µl	1000 µl of dilution A	200 µg/ml
E	1000 µl	1000 µl of dilution D	100 µg/ml
F	1000 µl	1000 µl of dilution E	50 µg/ml
G	1000 µl	0 µl	0 µg/ml
H	1000 µl	0 µl	0 µg/ml

Table 1. Preparation of diluted bovine serum albumin (BSA) standards for Bradford protein assay.

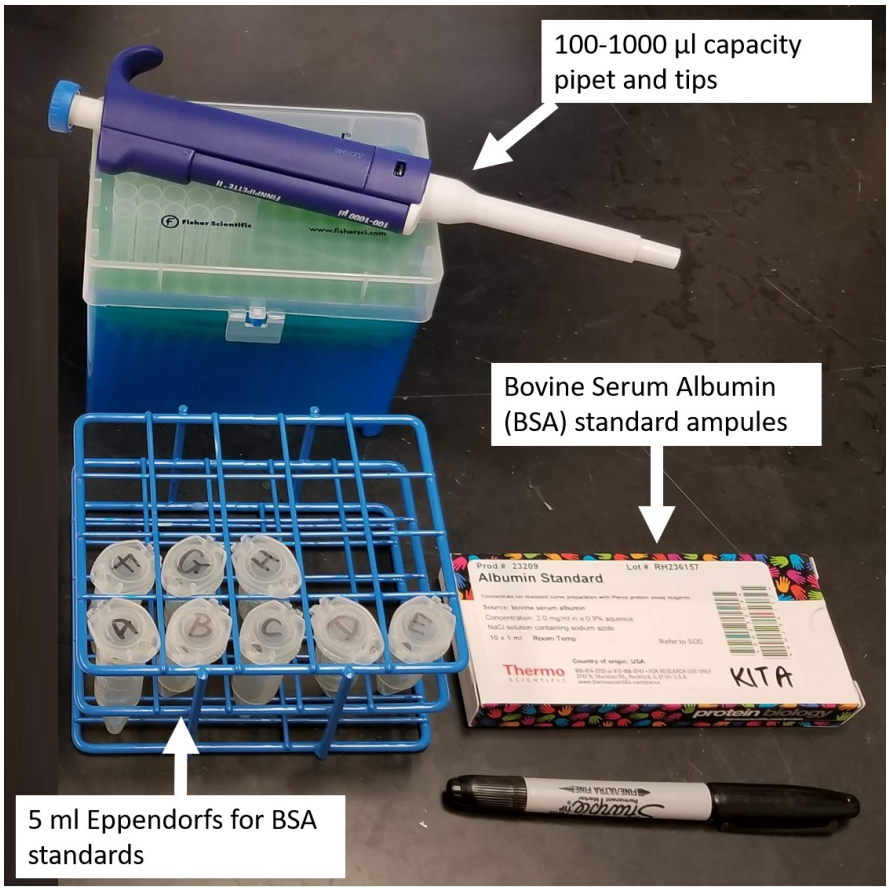

Fig. 7. Equipment needed for making BSA protein standards.

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10 **Prepare dye reagent.** Prepare 150 ml of Bio-Rad protein assay dye reagent from concentrate (keep refrigerated). Using a glass graduated cylinder, dispense 30 ml of the Bio-Rad protein assay dye and 120 ml of ultrapure water into a clean Erlenmeyer flask. Gently swirl to ensure thorough mixing. Cover with Parafilm until you are ready to load dye into the microplates.

11 **Prepare microplates.** Using a water-resistance marker pen, label the side of two polystyrene 96-well microplates, one with the letter "A" and one with the letter "B". *Note that for each set of 24 samples, you will need two microplates: plate A is for dilutions and plate B is for the Bradford assay.*

12 **Load samples.** All samples are loaded in triplicate in columns 2–10 of plate A (Fig 8). Using a 20 µl capacity pipette and one clean tip per sample well, dispense 5 µl of supernatant from the 2 ml sample Eppendorf tubes from step 8 into the appropriate microplate wells in plate A. Care should be taken to minimize bubbles forming in the solutions transferred in this and subsequent steps. It is beneficial to make a *96-well plate loading template* before you begin as a guide (Fig. 8). Leaving columns 11 and 12 empty as spares allows room for error, for example, if a mistake is made during pipetting or diluting samples. After loading all of the sample wells, return the 2 ml Eppendorf tubes to the -80 °C freezer, as these will be needed later for ash-free dry weight quantification.

Important: Do not load the standards into this plate (plate A). Standards will eventually be loaded into column 1 on plate B (see step 15).

Column number / Row letter	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Sample 1.1	Sample 1.2	Sample 1.3	Sample 2.1	Sample 2.2	Sample 2.3	Sample 3.1	Sample 3.2	Sample 3.3	Spare	Spare
B	Std B	Sample 4.1	Sample 4.2	Sample 4.3	Sample 5.1	Sample 5.2	Sample 5.3	Sample 6.1	Sample 6.2	Sample 6.3	Spare	Spare
C	Std C	Sample 7.1	Sample 7.2	Sample 7.3	Sample 8.1	Sample 8.2	Sample 8.3	Sample 9.1	Sample 9.2	Sample 9.3	Spare	Spare
D	Std D	Sample 10.1	Sample 10.2	Sample 10.3	Sample 11.1	Sample 11.2	Sample 11.3	Sample 12.1	Sample 12.2	Sample 12.3	Spare	Spare
E	Std E	Sample 13.1	Sample 13.2	Sample 13.3	Sample 14.1	Sample 14.2	Sample 14.3	Sample 15.1	Sample 15.2	Sample 15.3	Spare	Spare
F	Std F	Sample 16.1	Sample 16.2	Sample 16.3	Sample 17.1	Sample 17.2	Sample 17.3	Sample 18.1	Sample 18.2	Sample 18.3	Spare	Spare
G	Std G	Sample 19.1	Sample 19.2	Sample 19.3	Sample 20.1	Sample 20.2	Sample 20.3	Sample 21.1	Sample 21.2	Sample 21.3	Spare	Spare
H	Std H	Sample 22.1	Sample 22.2	Sample 22.3	Sample 23.1	Sample 23.2	Sample 23.3	Sample 24.1	Sample 24.2	Sample 24.3	Spare	Spare

Fig. 8. Example of a 96-well plate loading template. Each sample is loaded in triplicate.

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13 **Dilute loaded samples.** Using a 20 µl capacity multichannel pipette, and a disposable pipetting reservoir (Fig. 9), add 20 µl of ultrapure water to the sample wells of plate A. This procedure dilutes the corals samples 1:5 (determined as correct range via pilot trials, but could vary depending upon the coral species). *Alternative dilutions (i.e. 1:6, 1:7, etc.) can be achieved during this step by increasing or decreasing the volume of ultrapure water added.* After ultrapure water has been added to all wells, place plate A on a 96-well plate shaker for 1 minute to mix the sample and ultrapure water thoroughly.

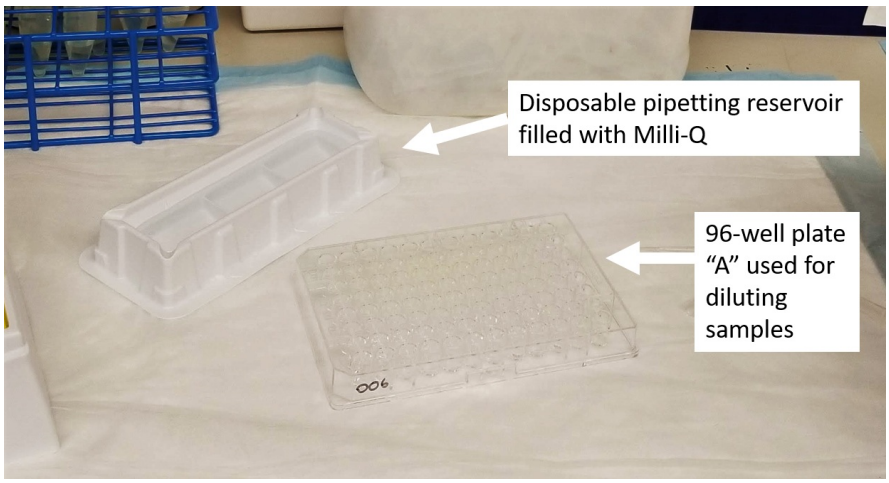

Fig. 9. Disposable loading tray filled with ultrapure water for diluting protein samples in plate A.

14 **Transfer samples from plate A to B.** Using a 20 µl capacity multichannel pipette, transfer 10 µl from each diluted sample well of plate A (i.e. columns 2-10, rows A-H) into the same wells of plate B.

15 **Load standards.** Using a 20 µl capacity pipette, dispense 10 µl of each BSA standard (Eppendorfs A - H) into column #1 of tray B. Do not add ultrapure water to these 12 wells. For convenience, load standard A into well 1A, standard B into well 1B, etc.

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16 **Load dye reagent.** Using a 200 µl capacity multichannel pipette, and a disposable loading tray, add 190 µl of 1X diluted dye reagent to every well of the 96-well microplate, starting with column 1. Use clean tips for every well. Record the time of dye addition.

Place plate B on a shake-plate for 1 minute to mix diluted samples/standards and reagent dye thoroughly. Incubate plate at room temperature for at least 5 minutes. Absorbance values will increase over time; samples should incubate for no longer than one hour.

After adding the dye, all wells should be varying intensities of blue color, with the exception of column 1, rows G and H (the blanks) which should be orange (Fig. 10)

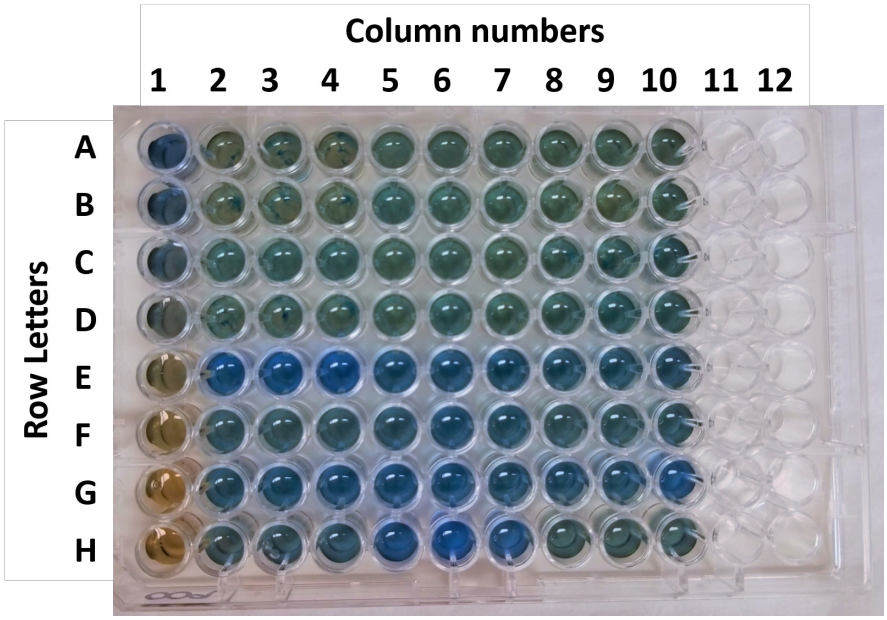

Fig. 10. Photo of plate "B" after the reagent dye has been added showing color change.

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17 **Pop bubbles.** Using a clean pipette tip per well, pop any air bubbles which have formed in the sample wells (Fig. 11).

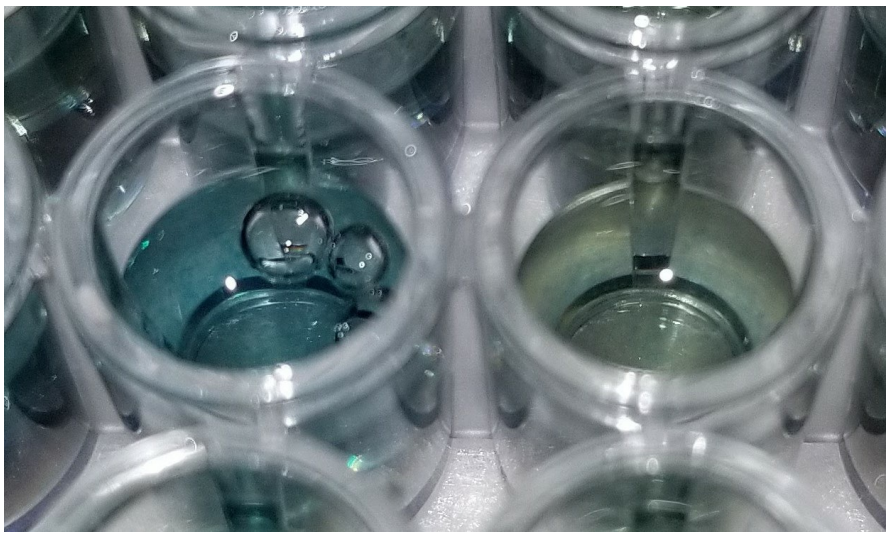

Fig. 11. Left: Well with air bubbles. Right: Well without air bubbles.

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18 **Absorbance spectrophotometry.** Measure the absorbance at 595 nm using a FlexStation® 3 Benchtop Multi-Mode Microplate reader. Manual with full instructions for using the SoftMax Pro software are available online, but briefly: Open *SoftMax Pro 5.4.1 > Protocol > Protein Quant > Bradford > Template* (create you template here) *> Okay > Read > Save As* and *Copy & Paste* into excel.

NOTE: This software automatically calculates the average absorbance of each sample using the triplicate wells, corrects for the two blank standards (cells G1 and H1), plots a standard calibration curve (Fig. 12) and calculates the protein concentration for each sample

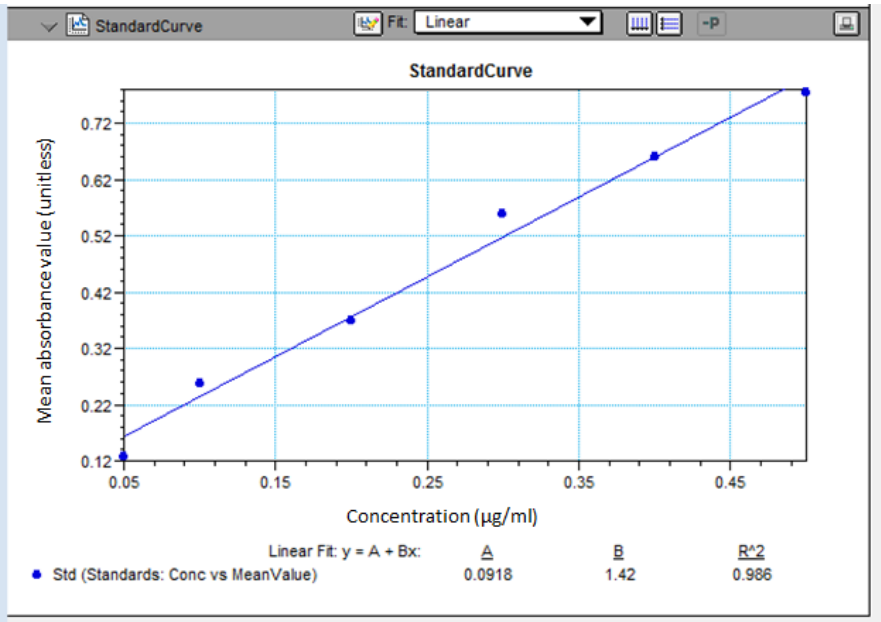

Fig 12. Example of the standard BSA curve produced by the SoftMax Pro software, with mean absorbance on the y-axis, and protein concentration on the x-axis.

The average concentration of protein for each sample is reported as the **Adjusted mean concentration (mg/ml).**

19 **Calculations.** The following equations are required in order to obtain the protein content (J) per sample.

(Eq. 1) Final volume of subsample (ml) = Volume of ground coral subsample (ml) + Volume of RIPA buffer added (ml),

where 1 g of ground coral paste is assumed to be equivalent to 1 ml

(Eq. 2) Weight of protein in subsample (mg) = Adjusted mean concentration (mg/ml) x Final volume of subsample (ml)

(Eq. 3) Energy content of protein in subsample (J) = Weight of protein in subsample (mg) x 23.9 J mg^{-1},

where the enthalpy of combustion for protein is -23.9 J mg^{-1} (Gnaiger and Bitterlich, 1984)

Gnaiger E and Bitterlich G (1984). Proximate biochemical composition and caloric content calculated from elemental CHN analysis: a stoichiometric concept. Oecologia, 62:289–298.

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20 Combine the remaining contents of the 15ml centrifuge tube (tissue and skeletal particles + white matrix) and 2 ml Eppendorf (soluble protein in RIPA) into pre-baked, pre-labeled aluminum pans. Make a control by dispensing 1000 µl of 1X RIPA into a pre-labeled aluminum pan. Place all the aluminum weighing pans in a 60 °C drying oven overnight.

The next day, remove pans from the oven and allow them to cool for 5–10 minutes before weighing. Weigh each dried pan on a balance. All pans are weighted in triplicate and recorded on a data sheet (Fig 13). Enter these values into excel and calculate the average of the three "*Dried Pan Weights*".

After weighing, place pans into a muffle furnace. Burn the dried sample at 450 ºC for 5 hours. Remove pans from the muffle furnace and allow them to cool for 5–10 minutes before weighing. Weigh each burnt pan on a balance. All pans are weighted in triplicate and recorded on the datasheet (Fig. 13). Enter these values into excel and calculate the average of the three "*Burnt Pan Weights*".

Sample ID	Dry Pan Weight (g)			Burnt Pan Weight (g)			Date/Time Out of Oven: Date/Time Out of Muffle: NOTES
	#1	#2	#3	#1	#2	#3	
CONTROL							

Fig. 13. Example of a dataset for recording the dried and burnt weights during the ash-free dry weight procedure.

To calculate protein energy content per gram of AFDW, use equations 4 and 5 below:

(Eq. 4) AFDW of the subsample (g) = [average CORAL *Dried Pan Weight (g)* - average CORAL *Burnt Pan Weight (g)*] [average CONTROL *Dried Pan Weight (g)* - average CONTROL *Burnt Pan Weight (g)*]

(Eq. 5) The total protein energy (J g^{-1}) = Energy content of protein in subsample (J) ÷ AFDW of the subsample (g)

If protein energy content per surface area is desired, use equations 6 and 7 below:

Note: Quantification of the coral surface area is outside the scope of this protocol, as there are several methods (foil-wrap, wax-dip, 3d-scan, etc.). This protocol assumes the operator already knows the surface area of the whole coral fragment that was ground into a homogenous paste at the start of this protocol. The surface area (SA) of the subsample used for soluble protein quantification is calculated using the following equation:

(Eq. 6) SA subsample (cm^2) = [wet weight of subsample (g) ÷ total wet weight of the ground coral fragment (g)] x SA whole colony (cm^2)

(Eq. 7) The total protein energy (J cm^{-2}) = Energy content of protein in subsample (J) ÷ SA subsample (cm^2)

Citation: Rowan Mclachlan, James Price, Kerri Dobson, Noah Weisleder, Andrea Grottoli (04/09/2020). Microplate Assay for Quantification of Soluble Protein in Ground Coral Samples. https://dx.doi.org/10.17504/protocols.io.bdc8i2zw

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