

Protein Extraction and Quantification

Application: Total soluble protein extracted from Cnidarian tissue (e.g. scleractinian corals, soft corals, and anemones) for multiple diagnostic assays (i.e. western blot, enzyme linked immunosorbent assay [ELISA], and Porphyrin Microplate Fluorescence Assay [[LINK to Porphyrin protocol](#)]).

Equipment & Supplies:

- 0.5 ml amber tubes
- 1.5 ml safe-lock microcentrifuge tubes. *Locking-cap microcentrifuge tubes prevent caps from popping open when samples are heated.*
- 2 ml cryovials containing frozen ground samples (Tissue homogenization [LINK](#))
- 6-aminocaproic acid 99+%
- air blow dryer
- benzamidine hydrochloride hydrate
- bovine serum albumin protein standard - BSA
- Coomassie destain [[LINK to solution recipe](#)]
- Coomassie stain [[LINK to solution recipe](#)]
- deferoxamine mesylate salt - DM
- denaturing buffer [[LINK to solution recipe](#)]
- dimethyl sulfoxide 99.9% ACS spectrophotometric grade - DMSO
- dithiothreitol - DTT
- brilliant Blue R 250
- ethylenediaminetetraacetic acid disodium salt dehydrate – EDTA
- freezer grip, e.g. rubber drawer liners cut into 5 in x 10 in swatches and folded over once (household goods store)
- freezer -80°C
- glacial acetic acid, A.C.S Plus
- hydrochloric acid technical grade - HCl
- methanol sequencing grade
- hemostat
- imaging software, e.g. Adobe Photoshop CS4 Extended version 11.0.2, NIH Image J for Mac OS X® or Windows operating system®, or Genetools®- analysis software for GeneSnap® (Syngene)
- 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)
- liquid nitrogen (LN₂) or Dry Ice
- LN₂ Dewar or container for Dry Ice
- microfuge with maximum *g* force 21,000 and maximum speed 15,000 rpm (e.g. MicroMax RF Thermo IEC)
- MilliQ water source
- Minicentrifuge (6 x 1.5ml rotor with 0.5ml adaptors), 6000rpm, 2000xg (rcf)
- micropipettors with 0.5-2.0 µl range (P2), 20-200 µl range (P200), and 100-1000 µl

- range (P1000) (e.g. Gilson Inc, Middleton WI)
- No. 2 pencil
 - P1000, P200, and P2 tips
 - phenylmethanesulphonylfluoride - PMSF
 - plastic container with top, no smaller than 100mm diameter
 - polyvinylpolypyrrolidone - PVPP
 - protease inhibitor cocktail for plants catalogue # p9599 (Sigma Aldrich, St. Louis MO)
 - rocking platform
 - sodium dodecyl sulfate – SDS
 - sorbitol
 - spatulas
 - Styrofoam container with tube inserts
 - submersible thermometer (0 – 100 °C)
 - TE-SDS [[LINK to solution recipe](#)]
 - tris (hydroxymethyl)aminomethane base - Tris
 - tube rack
 - vortex mixer
 - water bath set to 90°C to hold up to twelve 1.5 ml safe-lock tubes
 - water bath set to 65°C to hold up to twelve 1.5 ml and 0.5 ml microcentrifuge tubes
 - Whatman No. 5 filter paper 90mm to 150mm in diameter

Stock Solutions

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

Procedure

I. Tissue Homogenization ([LINK](#))

II. Protein Extraction ([Flow chart LINK](#))

1. On the day of extraction, freshly prepare denaturing buffer for 20 ml, weigh 121.14 mg of Tris and 74.4 mg of EDTA and mix with 16 ml of dH₂O in a 50 ml conical tube. Next, adjust pH of Tris -EDTA solution to 7.8 and then add 4 g of SDS. 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 µl of 1 mM sorbitol, 20 µl of 50 mM DM, 77.1 mg DTT, 200 µl DMSO and bring to a final volume of 20 ml with dH₂O. Finally, add 0.8 g of PVPP to the denaturing buffer.
2. Transfer cryovials containing frozen ground tissue samples from the -80°C freezer into LN₂ or dry ice until ready for use.
3. Prepare extraction tubes by adding the following amounts of protease inhibitors

to the inside caps of 1.5 ml safe-lock microcentrifuge tubes: 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.

4. Chill spatulas in LN₂ before picking up frozen ground tissue samples to prevent tissue from thawing and adhering to spatulas.
5. Retrieve ground tissue samples from LN₂ using a hemostat.
6. Transfer 150 to 250 μ l of frozen ground tissue sample using a chilled spatula into the graduated 1.5ml safe-lock eppendorf tubes and quickly add 1 ml of denaturing buffer followed by closing caps immediately. 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. NOTE: See troubleshooting & optimization section below.
7. Vortex samples for 30 s and gently invert tubes repeatedly for 1 minute so that the denaturing buffer completely interfaces and mixes with the sample.
8. Incubate samples in a 90°C water bath for 3 min.
9. Retrieve samples from the water bath and vortex for 30 s.
10. Repeat steps 8 and 9 twice and then place tubes at room temperature (23-25°C) for 5 min.
11. Spin down samples for 15 min at 20,800 \times *g*. Three phases are visible after centrifugation as shown in the protein extraction flow chart below.
12. Carefully aspirate the middle phase of sample extract using a micropipette with a 20-200 μ l range (e.g. Gilson P200). The middle phase should contain the soluble protein fraction free of mucus and insoluble particles. NOTE: Change micropipette tip if an additional aspiration is needed for the same sample to insure that the mucus adhering to the tip pulled from the top phase will not contaminate the middle phase.
13. Aliquot a sufficient amount (50 μ l – 100 μ l) of protein extract per tube to do one assay. Avoid repeated freeze/thaw of extracts to prevent protein degradation and maintain protein stability. The porphyrin assay uses the whole liquid phase (top and middle) since mucus contamination does not interfere with this assay. Other assays, may call for clean extracts (middle phase only) if the mucus will interfere with detection of the assay (e.g. western blot and ELISA). If the assay requires clean extracts, do not allow pipette tip to touch the inside of the tube to minimize chance of contamination. Prior to storing the protein extracts in the -80°C freezer, remove 3 μ l from each protein extract for protein quantification as described in the next section.

III. Protein Quantification ([Flow Chart LINK](#))

Spot Blot

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.
- Heat BSA standard dilutions and frozen sample extracts to 65°C for 5 min. Spin standards and sample extracts quickly and then vortex.
 - Micropipette 1 μ l of each BSA standard and sample in triplicate vertically (columns) and uniformly on Whatman No. 5 circular paper. Under each column of BSA standard spots, mark a line with a pencil, indicating a reference point. This prevents spotting onto a standard or sample that has dried and is not visible.
 - Air dry spot blot for 30 min or use an air blow dryer to dry in less than 1 min.
 - Place spot blot into Coomassie stain in a plastic container with a lid for 15 min on a rocking platform at room temperature.
 - While holding the spot blot in place, decant stain and rinse container once with dH₂O. Do not rinse the spot blot directly.
 - 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.
 - Air dry blot for 1 hr, or blow dry blot in less than 1 min.

Spot Densitometry

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

- Place spot blot within the G-Box unit onto the gel box.
- Place a clean glass plate on top of the spot blot to keep it from curling.
- Adjust the parameters (iris, zoom, fine focus, brightness, contrast, and gamma) using the software provided by the imaging system (e.g. GeneSnap®).
- Select “upper-white” and “no binning” before acquiring the image according to the manual’s instructions.
- Save both sgd file format and TIFF (.sgd to be used in Genetools® and Tagged Image File Format to be opened using most imaging software).

Protein Determination

Imaging System analysis software (e.g. GeneTools®)

- Select the following options under the “General” tab: “Spot Blot, Circle, Absorption, and Leave spots unchanged”. Select “OK”.
- Edit “Quantity Calibration” parameters by selecting curve type and give the units (μ g/ μ l).
- Manually insert the first trace circle around the first BSA standard spot 1 by double clicking on the center of this spot. Select “OK”.
- 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 circle, hold down cursor and drag.
- Enter the known concentration of this standard under “Quantity.”
- Repeat step 16 for the rest of BSA standards and sample spots. Under “Spots” on the top task bar, select all same size spots.
- 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 on background closest to spots. Do not get any portion of the actual spot into the background correction boxes. Background should be conducted on all standards and sample spots.

21. Data, linear regression, and unknown sample quantities (μg) are generated automatically, which can be exported into a Microsoft Excel® or Word document®.

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).

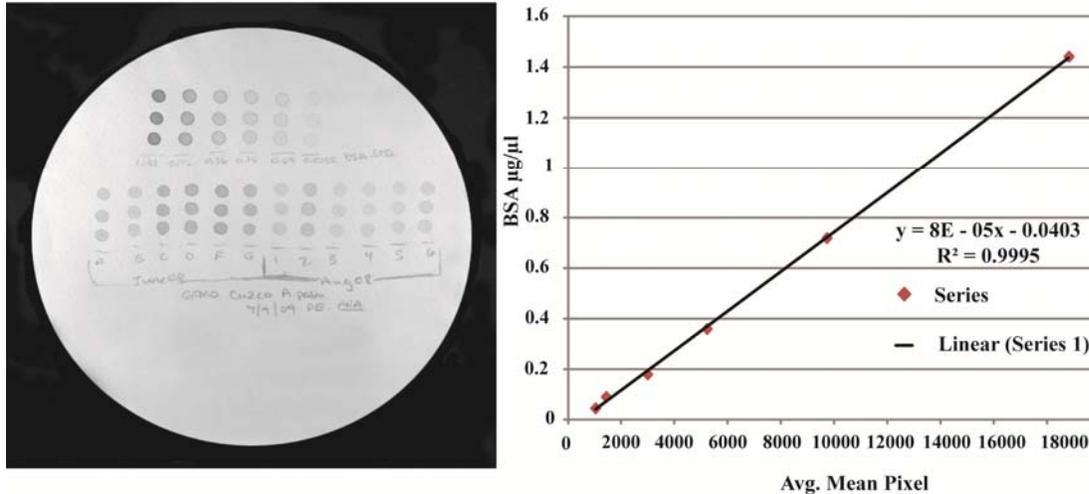
Alternative programs to calculate density of protein spots are Adobe Photoshop® and NIH Image J for Mac OS X or Windows operating system. NIH Image J for MAC and PC operating systems can be downloaded along with instructions from the website <http://rsb.info.nih.gov/nih-image/>(Rasband 2011). Below is a short protocol to determine protein concentration from the spot blot using Adobe Photoshop CS4 Extended, version 11.0.2.

Adobe Photoshop CS4 Extended, version 11.0.2

1. Open TIFF (Tagged Image File Format) and invert 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 lines.
3. Use the elliptical marquee tool to select a region within a desired spot and the mean pixel average, standard deviation, median and pixels should be displayed in the histogram box. This data cannot be exported into a file and should be noted by the user for each triplicate.
4. A standard curve can be generated using graphing software such as Microsoft Excel or SigmaPlot. The mean pixel values are averaged for the standard spots for each triplicate set and plotted against the known BSA standard protein concentration of the dilution series on a scattered x-y graph with a linear trend line. Interpolation is used to calculate the concentrations of unknown samples from the linear regression. ([LINK](#) to Linear regression explanation)

IV. Expected Results

1. Spot blot and Standard Curve



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.

2. Protein Quantification

Total Soluble Protein Range from Select Cnidarians. * Each *N. vectensis* was weighed on a microgram scale. A whole animal was used per extract. A key for color abbreviations: 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)	Color of protein extract	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	1.2-1.4
<i>Montastraea annularis</i>	150	500	MY, Green foam on 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

Considerations & Caveats

Time Considerations

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

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 2 hr for 20 samples. NOTE: 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 a digital image capture instrument 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.

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 (polyvinylpolypyrrolidone) is used to bind polyphenolic compounds found in cnidarian samples that contain symbiotic algae (zooxanthellae).
- SDS (sodium dodecyl sulfate) is an anionic detergent used to facilitate the denaturation of proteins by disrupting their non-covalent bonds.
- Sorbitol is a sugar alcohol used as a cryoprotectant.
- DM (deferroxamine mesylate salt) is an iron chelating agent.
- DTT (dithiothreitol) 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 (dimethyl sulfoxide) is an organosulfur solvent that facilitates protein solubilization.
- Protease inhibitors target specific *protease classes*:
 - 5µM 6-aminocaproic acid: *Lysine proteases*
 - 2mM benzamidine: *trypsin and serine proteases*
 - 2mM PMSF: *serine proteases*

- 0.01% protease inhibitor cocktail: *serine, cysteine, aspartic, metalloproteases, and aminopeptidases.*

References & Links

Downs, C.A. (2005) Cellular diagnostics and its application to aquatic and marine toxicology. In: G. Ostrander (ed) *Techniques in aquatic toxicology*. 2. CRC Press, Inc., Boca Raton, pp 301-313

Ghosh, S., Gepstein, S., Heikkila, J.J., *et al.* (1988) Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissues. *Analytical Biochemistry*, 169, 227-233.

Rasband, W.S. (2011) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011.

Sokal, R.R. and Rohlf, F.J. (1995) Linear Regression. In: *Biometry the principles and practice of statistics in biological research*, 3rd edn. pp. 452-455. W.H. Freeman & Co., New York.

([LINK to Protein Extraction/Quantification TM-###](#))

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