Porphyrin Microplate Fluorescence Assay

Application: To detect total porphyrin from Cnidarian tissue extracts.

Equipment & Supplies:

- 1.5 ml amber microcentrifuge tubes
- 12 N hydrochloric acid (HCl)
- 15 ml glass vials with screw on cap wrapped in aluminum foil
- 9N HCl
- 96 well optical bottom black plate & cover
- Analysis software for Scanning Spectrofluorometer- e.g. SpectraMax Pro
- Analysis software for filter-defined spectrofluorometer - e.g. KC4 microplate data analysis software (Biotek®)
- Ethylenediaminetetraacetic acid disodium salt dehydrate – EDTA
- Filter-defined spectrofluorometer - e.g. Synergy HT microplate reader
- Milligram balance
- Micropipettors with 10-100 µl range (P100), 20-200 µl range (P200), and 100-1000 µl range (P1000) (e.g. Gilson Inc, Middleton WI)
- Minicentrifuge (6 x 1.5 ml rotor with 0.5 ml adaptors), 6000rpm, 2000 *xg* (rcf)
- P1000, P200, and P100 tips
- Protoporphyrin IX - PP IX (e.g. Frontier Scientific)
- Refrigerator for storage at 4°C
- Sample protein extracts (*see Protein Extraction and Quantification protocol **LINK***)
- Scanning spectrofluorometer - e.g. SpectraMax
- Sodium dodecyl sulfate - SDS
- Statistical software- e.g. Microsoft Excel® or SigmaPlot®.
- Tris (hydroxymethyl)aminomethane base - Tris
- Tris EDTA SDS – TE-SDS (**LINK** to Solutions)
- Timer
- Vortex mixer
- Water bath up to 65°C

Methods:

I. Tissue Homogenization (Virtual Web Protocol LINK)

II. Protein Extraction and Quantification (Virtual Web Protocol LINK)

III. Porphyrin Standard Preparation

Porphyrins are light sensitive and consequently this assay must be conducted in a dark room with indirect light to prevent photo-degradation of standards and samples to be analyzed for porphyrin. Amber or tinfoil wrapped glass vials should be used to store porphyrin

solutions to prevent photo-oxidation.

1. Weigh 1.125 mg of protoporphyrin (PP IX) standard with a milligram balance and place into 1.5 ml amber microcentrifuge tube or glass vial.
2. Reconstitute PP IX with 1 ml 12N HCl to 2 µmol / 1 ml. Vortex for 1 min.
3. Prepare 100 nmol ml stock solution of PP IX while adjusting the final concentration of HCl to 1N. Dissolve 1.125 mg of PPIX in 1 ml of 12N HCl. Next, dilute the 2 µmol / 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.
4. Store stock solutions of standard in capped vials at 4°C.
5. Prepare 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. Make a set of porphyrin standards: 5000, 3500, 3000, 2500, 2000, 1000 and 500 fmol in a standard volume of 100 µl for each (e.g., 5000 fmol / 100 ul; 3500 fmol / 100 ul)
6. Store standards at 4°C no longer than 6 months as per manufacturer's recommendation.
7. 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.

Acidification is used in this assay to oxidize porphyrinogens to porphyrins for the 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).

IV. Porphyrin Microplate Fluorescence Assay (Flow Chart – LINK)

Conduct the assay in a dark room with indirect light. As long as the assay samples and standards are not exposed to direct light and only a short time with indirect light, photo-degradation should be minimal.

Fluorescence detection with a filter-defined spectrofluorometer (Synergy HT)

1. Set up a protocol for the microplate reader (e.g. KC4 software).
    i. Fluorescence
    ii. 400 ± 15 nm bandwidth excitation filter
    iii. 600 ± 20 nm bandwidth emission filter
    iv. Pre-read blank plate
    v. Read wells A1-H12
    vi. Photomultiplier (PMT) sensitivity should be optimized for the instrument

and assay samples.

2. Define the plate layout with standards, samples, and assay controls.
3. Read blank plate. Always read blank plate before plating samples and controls onto microplate.
4. Plate is ready to read and should take no longer than 15 min depending on whether the plate is full and on the number PMT sensitivity settings being tested.
5. Calculate the amount of TE-SDS buffer needed after 10 µg of sample TSP and or standard is added to each well for a total volume of 320 µl. Pipette the calculated amount of TE-SDS buffer into each well (*see microplate layout below*).
6. Prepare a 7 point calibrant curve in triplicate from 5,000-500 fmol using 100 µl from the porphyrin standard working solution described in step 5 of the Prophyrin Standard Preparation method outlined earlier.
7. Thaw frozen sample protein extracts to 65°C for 5 min, spin quickly, vortex and pipette 10 µg in triplicate into wells with pre-calculated amounts of TE-SDS buffer to total 320 µl.
8. Read blank plate on spectrofluorometer before adding HCl.
9. Add 40 µl of 9N hydrochloric acid to each well to bring the total volume to 360 µl. Cover the plate and incubate at room temperature for 15 min prior to reading plate on a spectrofluorometer. The microplate should consist of a 7 point calibrant curve of porphyrin standards 500 – 5,000 fmol, assay control of extraction buffer, and samples.

MICROPLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	EB	s1	s1	s1	2	s9	s9	s9	5	s18	s18	s18
B	EB	s2	s2	s2	3	s10	s10	s10	5	s19	s19	s19
C	EB	s3	s3	s3	3	s12	s12	s12	6	s20	s20	s20
D	1	s4	s4	s4	3	s13	s13	s13	6	s21	s21	s21
E	1	s5	s5	s5	4	s14	s14	s14	6	s22	s22	s22
F	1	s6	s6	s6	4	s15	s15	s15	7	s23	s23	s23
G	2	s7	s7	s7	4	s16	s16	s16	7	s24	s24	s24
H	2	s8	s8	s8	5	s17	s17	s17	7	s25	s25	s25

Porphyrin Microplate Layout. Numbers 1-7 highlighted in blue represent the porphyrin standards in triplicate from 500 fmol to 5,000 fmol. Samples are designated here as s1-s25 and the assay control as "EB" for extraction buffer (e.g. TE-SDS).

Porphyrin Quantification

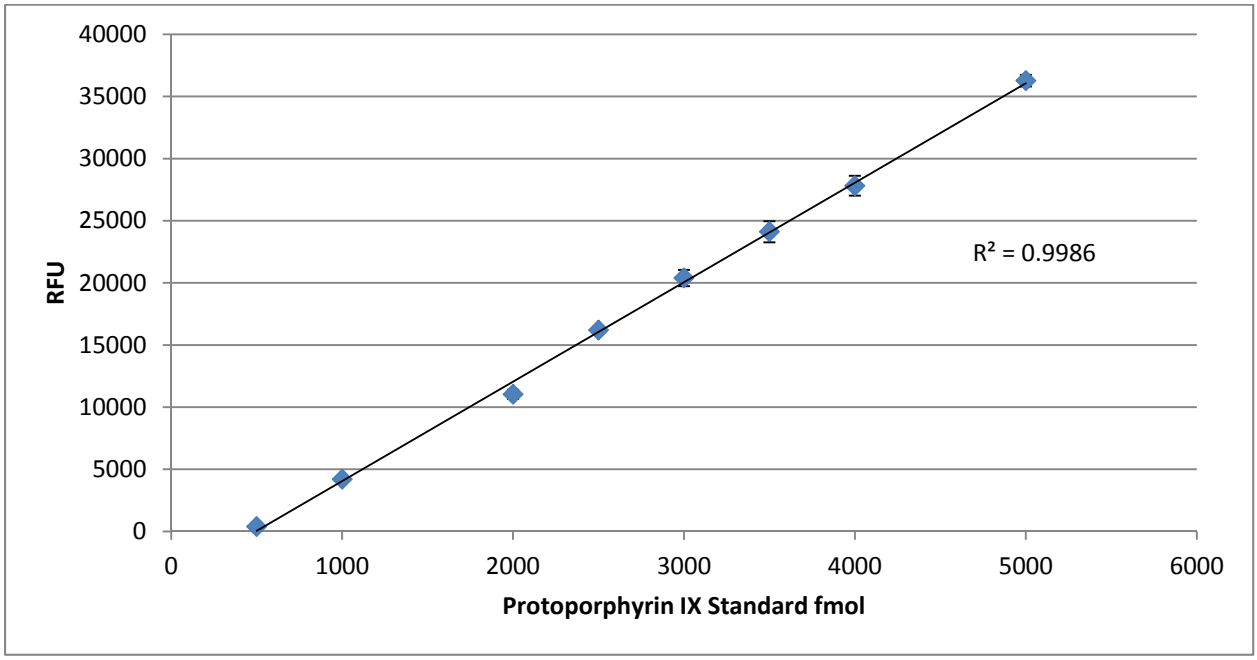
10. Export raw data into an Excel spreadsheet or text file that can be transferred later to graphing software like SigmaPlot.
11. Select data from the data set (assorted by PMT sensitivity) that has the highest relative fluorescence units (RFUs) for the porphyrin standards without reaching

saturation. Saturation may be denoted by some character like an exclamation mark.

12. Find the average of each of the standard triplicates and unknown sample triplicates. 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.
13. Use slope-intercept formula to determine porphyrin concentration for each sample (Sokal & Rohlf 1995). (**LINK** http://graphpad.com/curvefit/linear_regression.htm)

V. Expected Results

1. Protoporphyrin IX Standard Linear Regression



2. Porphyrin Quantification

Species	fmol/µg (TSP)	SE	Origin
Aiptasia pulchella	86.8	39.0	LC
	194.3		
	211.4		
Montastraea annularis	199.2	40.8	LC
	85.1		
	69.8		
Porites divaricata	128.4	27.4	LC
	79.7		
	174.7		

Fungia fungites	83.7	12.6	LC
	114.5		
	72.1		
Acropora chesterfieldensis	94.2	16.1	LC
	47.7		
	97.8		
Acropora millepora	118.5	12.7	LC
	144.0		
Nematostella vectensis	33.6	2.2	LC
	28.1		
	35.5		
Leptogorgia virgulata	44.9	4.4	F
	29.7		
	35.6		
Acropora palmata	66.3	12.5	F
	135.0		
	100.5		
	118.5		
	79.8		
Montastrea faveolata	309.0	74.2	F
	399.1		
	145.7		

Reference samples of 10 selected cnidarians from different habitats in the field (F) and samples that have been taken from the field and cultured in the lab (LC) were evaluated for nominal porphyrin levels. Shown here is the total porphyrin in fmol / µg of total soluble protein (TSP).

Considerations & Caveats:

A. Time

PP IX standard preparation can take up to 30 min. It takes 25 min (25 samples) to incubate frozen samples for 5 min in a 65˚C water bath and dilute standards and samples with appropriate amounts of TE-SDS. Plating, incubation with HCl, and reading the microplate can take up to 45 min.

B. Plating

The TE-SDS buffer has a high concentration of SDS which can create a lot of bubbles in the wells. The bubbles 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 plunger.

References & Links

Rimington, C. (1960). Spectral-absorption coefficients of some porphyrins in the soret-band region. *Biochem Journal* 75: 620-623

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.

Zhang, Y., Li M.X., Lü M.Y*., et al.* (2005). Anion chelation-induced porphyrin protonation and its application for chloride anion sensing. *The Journal of Physical Chemistry A* 109: 7442-7448

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