Fluorimetric Enzyme Assay Protocol for Marine Samples

(Modified from S. Schmidt Lab/M. Weintraub U. of Colorado Boulder, August 2005; Suding Lab, January 2008; and Saiya-Cork, K. R., R. L. Sinsabaugh, and D. R. Zak. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biology & Biochemistry **34**:1309-1315.)

Sample preparation:
Each day, there will be two replicate ocean water samples. You will measure enzyme activities in 0.22 μm-filtered (F-fraction), 2.7 μm-filtered (S-fraction), and unfiltered (U-fraction) water for each replicate (a total of 6 measurement samples).

To obtain the filtered water, push 50 ml seawater through either a 0.22 μm syringe filter (F fraction) or 2.7 μm syringe filter (S-fraction) and into a clean bottle or tube.

You will also need sterilized, filtered (0.22 μm) seawater, which can be made in a large batch and used over time as long as it's not contaminated.

Substrate solutions:

Enzyme	Substrate
β-Glucosidase (BG)	4-Methylumbelliferyl β-D-glucopyranoside 200 μM = 1.02 mg/15 ml
β-N-acetylglucosaminidase (NAG)	4-Methylumbelliferyl N-acetyl-β-D-glucosaminide 400 μM = 2.28 mg/15 ml
Leucine aminopeptidase (LAP)	L-Leucine-7-amido-4-methylcoumarin hydrochloride 400 μM = 1.95 mg/15 ml Heat and stir to dissolve
Alkaline phosphatase (AP)	4-Methylumbelliferyl phosphate 1000 μM = 3.84 mg/15 ml
MUB standard stock solution	100 μM 4-Methylumbelliferone 1.76 mg/100 ml Heat and stir to dissolve
AMC standard stock solution	100 μM 7-Amino-4-methylcoumarin 1.75 mg/100 ml Heat and stir to dissolve

Make substrate and fluorescent standard solutions **one day ahead** in 125 ml **amber glass bottles** using sterile DI water and a microbalance. Store solutions in the 4°C refrigerator. Substrates are in freezer. Remake substrate solutions **every week**. Remake standard stock solutions **every 2 weeks**. Standards should be **diluted to 10 μM every day** by combining 0.5 ml stock solution with 4.5 ml sterile DI water.

Each plate is used to assay one enzyme and sample, so label each plate with the enzyme name. There is one additional control plate which should also be labeled as CON.

Assay Set-up
Add 200 µl of sample:

1	2	3	4	5	6	7	8	9	10	11	12
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2
F1	F1	F2	F2	S1	S1	S2	S2	U1	U1	U2	U2

Next add 50 µl diluted standards and appropriate substrates as follows:

1	2	3	4	5	6	7	8	9	10	11	12
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub
Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub	Stan	Sub

All wells should now contain 250 µl. Use AMC as the standard for LAP. Use MUB as the standard for BG, AP, and NAG. Add substrates and standards from left to right across plate and change tips if they contact the sample.

Then create a plate for controls with 200 µl of the following samples (Ster indicates sterile seawater):

1	2	3	4	5	6	7	8	9	10	11	12
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	
Ster	Ster	Ster	Ster	Ster	F1	F2	S1	S2	U1	U2	

Add 50 µl of the following substrates or DI water:

1	2	3	4	5	6	7	8	9	10	11	12
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	
BG	AP	NAG	LAP	DI	DI	DI	DI	DI	DI	DI	

Use new tips for each column.

Reading Plates
Read plates at 360 nm excitation and 460 nm emission at time zero and 30 minute intervals for 3 hours. Save your data in the appropriate Experiment files in the Gen5 software. Name each plate as follows:
Date Enzyme Time SampleNumbers

Date should be in YYMMDD or YYMMDD HH:MM format. Putting hours and minutes is optional and not necessary unless you are doing multiple samples in a single day. If you use hours and minutes, be sure to use military time (i.e. 13:30 instead of 1:30pm).

Enzyme should be BG, AP, NAG, or LAP

For example, for the assay plates:
080718 08:30 NAG 1.0 17-18
would be the 1 hour time point for NAG enzyme samples 17 and 18 taken on July 18, 2008 at 8:30 am.

For the control plates:
080718 08:30 CON 1.0 17-18

Activity ($\mu mol\ h^{-1}\ L^{-1}$) = slope of Concentration versus time in hours

Concentration = Raw Activity × 2.5 =
(Assay – Substrate Control) / {[(Standard – Water) / (10μmol/L × 0.00005L)] × 0.0002L}

Assay = Assay plates cols 2, 4, 6, 8, 10, 12
Substrate Control = Control plate cols 1-4
Standard = Assay plates cols 1, 3, 5, 7, 9, 11
Water = Control plate cols 6-11

Data Processing
In the Gen5 software for the plate reader, select the plates you want to export and click on "File Export" in the "Plate" menu. This should generate a text file containing the exported plates, including the plate ID and the average fluorescence value for each column of 8 wells. The file should appear on the Desktop with today's data as the filename (YYMMDD.txt).

Move the file from the Desktop to the Newport Time Series folder. All files you want to use should be in this folder.

Open the Windows command line (Start → Run → cmd).

At the command prompt, type:
perl OutputTimeSeries.pl

This will open a program that processes the raw data file from the plate reader and feeds it into a statistical and graphing program called R. You can also use the program to analyze and graph

plate reader files that have already been processed. The program will ask you the following questions about how you want to process the data and what statistics you want:

1) "Enter a filename to process from the plate reader or press 'Enter' to continue:"
If you have a new raw data file from the plate reader, enter its filename here. If you want to use an existing file that has already been processed, just press Enter without entering a filename.

2) "Enter 'y' to append data to an existing file or load an existing file.
Enter 'n' to create a new output file. (y/n)"
If you've analyzed data before and just want to add the new data to it, then say yes. If you already have a processed file you want to analyze, also say yes. If you want to create a new output file, then say no. Be careful not to append plates that are already present in the datafile; this will cause errors in the analysis.

If you say yes, the program will ask:
3) "What is the filename to load or append to?"
Enter the filename you want to analyze and/or add data to. If you did not enter a filename in step 1, then the file you request in this step (#3) will be loaded for further analyses and nothing will be appended.

If you say 'n' to create a new output file, the program will process your raw data file and output the results in a new text file with the same name as your input file and the suffix .out.txt.

Now the program will process your raw data and/or load the files you need to conduct statistical analyses.

4) "Show all regression plots? (y/n)"
Enter yes to see all the regression plots for the entire data file. Warning: this could be a very large number since each date usually generates 24 plots.

If you respond no, the program will ask:
5) "Enter number of dates to view regression plots:"
Enter an integer number and you will be able to view regressions from that many of the most recent dates. You can enter zero to suppress output of the regression plots.

6) "Show all timeseries dates? (y/n)"
Enter yes to see mean enzyme activities plotted for all of the dates, water fractions, and enzymes in the input data file.

If you respond no, the program will ask:
7) "Enter number of dates to view time series:"
Enter an integer number and R will output timeseries plots for that number of the most recent dates.

The plots will be output to a pdf file on the desktop called TimeSeries.pdf.

If there are problems with the input data (i.e. empty plates, incorrectly formatted plate IDs, etc.), the program will generate errors and may not run to completion. If there are errors, try to figure out what is causing them and then fix the problem. This may require editing plates in Gen5 and re-exporting the data or editing the processed data file, for example.

You should start with an existing file that has processed, quality-controlled data and only append to it once you are sure the new data are correct. Therefore, it's probably best to answer no to question #2, check all the data and plots, and then re-run the program with a yes answer to #2 and append to the existing file in question #3.