Fluorimetric Enzyme Assay Protocol for Streamwater

(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.)

Reagents:

50 mM Tris buffer, pH 7.5	**50 mM phosphate buffer, pH 7.5**
3.028 g Tris base	1.151 g $NaH_2PO_4 \cdot H_2O$
19.6 ml 1M HCl	2.365 g Na_2HPO_4
Adjust to pH 7.5 with 1M NaOH or HCl	Adjust to pH 7.5 with 1M NaOH or HCl
Bring to 500 ml with ultrapure water	Bring to 500 ml with ultrapure water

1.0 M NaOH
4 g NaOH pellets
100 mL DI water

Substrate solutions (200 µM unless otherwise noted):

Enzyme	**Substrate**
β-Glucosidase (BG)	4-Methylumbelliferyl β-D-glucopyranoside 6.77 mg/100 ml
β-N-acetylglucosaminidase (NAG)	4-Methylumbelliferyl N-acetyl-β-D-glucosaminide 7.59 mg/100 ml
Leucine aminopeptidase (LAP)	L-Leucine-7-amido-4-methylcoumarin hydrochloride 6.5 mg/100 ml
Alkaline phosphatase (AP)	4-Methylumbelliferyl phosphate 5.12 mg/100 ml
MUB standard	100 µM 4-Methylumbelliferone 1.76 mg/100 ml Dissolve in 1 ml acetone first
AMC standard	100 µM 7-Amino-4-methylcoumarin 1.75 mg/100 ml Dissolve in 1 ml acetone first

Make substrate and fluorescent standard solutions in 125 mL amber glass bottles using sterile water. Store solutions in the 4°C refrigerator. Substrates are in freezer. Remake solutions ~every 2 weeks or as needed. Standards should be diluted to 10 µM before use.

Label plates with the enzyme names.

For an assay with more than three samples, multiple plates for each substrate will be necessary (e.g. LAP 1, LAP 2, LAP 3…). At least 3 samples can be run per plate, so running 12 samples would require 4 plates for each assay.

Assay Set-up
Using 8-channel pipettor (and wide-mouth tips if necessary; cut off tip ends with scissors), pipette 175 µl of the sample into the 96 well plates, keeping sample well mixed. 1, 2, 3, etc. are the different samples. Note on datasheet which sample is which. All assay plates should be pipetted as follows, leaving columns 1-3 empty:

1	2	3	4	5	6	7	8	9	10	11	12
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3

or

1	2	3	4	5	6	7	8	9	10	11	12
none	none	none	1	1	1	3	3	3	5	5	5
none	none	none	1	1	1	3	3	3	5	5	5
none	none	none	1	1	1	3	3	3	5	5	5
none	none	none	1	1	1	3	3	3	5	5	5
none	none	none	2	2	2	4	4	4	6	6	6
none	none	none	2	2	2	4	4	4	6	6	6
none	none	none	2	2	2	4	4	4	6	6	6
none	none	none	2	2	2	4	4	4	6	6	6

Remember to change or use appropriate data transformations in Gen5 software.

Next add the following amounts (µl) of ultrapure water:

1	2	3	4	5	6	7	8	9	10	11	12
225	175	175	50			50			50		
225	175	175	50			50			50		
225	175	175	50			50			50		
225	175	175	50			50			50		
225	175	175	50			50			50		
225	175	175	50			50			50		
225	175	175	50			50			50		
225	175	175	50			50			50		

Now add 25 µl buffer to every well. Add Tris buffer to the AP and LAP plates, and phosphate buffer to the BG and NAG plates.

Next add the following amounts of diluted MUB standard (use AMC instead for the LAP assay):

1	2	3	4	5	6	7	8	9	10	11	12
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	

Finally, add the following amounts of substrate:

1	2	3	4	5	6	7	8	9	10	11	12
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50

All wells should now contain 250 µl.

Incubate plates at room temperature for 3-6 hours.

Assay Termination
Add 10 µL of 1 M NaOH to each well of all plates, noting time. Note: this is not done in advance. Add the NaOH to a set of plates for one assay only, then read those plates, then add the NaOH to another set and read those, etc.

Reading Plates
Read fluorescence plates at 360 nm excitation and 460 nm emission. Save your data in the appropriate Experiment files in the Gen5 software.

Activity (µmol h^{-1} L^{-1}) = (NFU / Standard FU) × (2.857 / hours) =
{NFU / [(Standard FU / (10 µmol/L × 0.00005 L)]} / (0.000175 L × hours)

NFU = net fluorescence units =
{(Assay – Sample) / [(Quench Control – Sample) / Standard]} – Substrate

Assay = Cols 6, 9, 12
Sample = Cols 4, 7, 10
Quench Control = Cols 5, 8, 11
Standard = Col 2
Substrate = Col 3