Fluorimetric and Oxidative Enzyme Assay Protocol

(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:

1.0 M NaOH
4 g NaOH pellets
100 mL DI water

50 mM sodium acetate buffer, pH 5.0 (can make a 10X stock solution)
6.804 g sodium acetate trihydrate
1.1 ml glacial acetic acid (add more to make pH = 5)
1 L sterile 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-Xylosidase (BX)	4-Methylumbelliferyl β-D-xylopyranoside 6.17 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 (dissolve in 0.5 ml acetone first)
Acid phosphatase (AP)	4-Methylumbelliferyl phosphate 5.12 mg/100 ml
Polyphenol oxidase (PPO) Peroxidase (PER)	25 mM L-dihydroxyphenylalanine (DOPA) **WARNING:** L-DOPA is hazardous; wear gloves. 493 mg/100 ml For soils also add: 50 mM EDTA (disodium, dihydrate) 1.861 g/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 DI water and a microbalance. Preheat water for L-DOPA solution in microwave. 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.

Label fluorescence (black) and colorimetric (clear) plates with the enzyme names.

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

Sample Preparation
If the dry weight of the sample is unknown, obtain fresh soil or litter samples and split each into two parts.

Weigh one part, record mass, place in tared coin envelope or soil tin and dry at 60-105°C to constant weight (2 days). Record dry mass.

Weigh other part (1-2 g wet weight), record mass, and place in labeled 500 ml container. Add 125 ml acetate buffer and blend on highest speed for 1 minute to make a slurry. If the dry weight of the sample is already known, it can be added directly to the 500 ml container. **Do not let samples sit in slurries for more than 30 minutes!**

Rinse blender with DI water between samples.

Assay Set-up
Using multichannel pipettors and wide-mouth tips (cut off tip ends with scissors if necessary), pipette 200 µl of the soil slurry into the 96 well plates, keeping slurry well stirred with a stir bar/plate. 1, 2, and 3 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

Next add the following amounts (µl) of sodium acetate buffer:

1	2	3	4	5	6	7	8	9	10	11	12
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		

Next add the following amounts of MUB (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.

For the PPO and PER plates add the following amounts (µl) of sodium acetate buffer:

1	2	3	4	5	6	7	8	9	10	11	12
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		

Then 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	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, except column 3 which is empty.
For PER plates only, add an additional 10 µl 0.3% H_2O_2 solution to every well.

Incubate fluorimetric and oxidase plates at room temperature. Incubation times will depend on your sample type.

Assay Termination
Add 10 µL of 1 M NaOH to each well of all fluorescence plates (NOT oxidase 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.

Wipe any condensation from the bottoms of the clear plates with a paper towel before reading. The data for these plates will not be accurate unless you wipe the plate bottoms off first.

Reading Plates
Read fluorescence plates at 365 nm excitation and 450 nm emission. Read PPO plates at 460 nm absorbance. Save your data.

Fluorescence Activity
Activity (µmol h^{-1} g^{-1}) = (NFU / Standard FU) × 0.3125 / (DW × hours) =
{NFU / [Standard FU / (10 µmol/L × 0.00005 L)]} / [0.0002 L × (DW g / 0.125 L) × hours]

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

DW = dry weight of soil sample contained in 125 ml buffer

Assay = Cols 6, 9, 12
Sample = Cols 4, 7, 10
Quench Control = Cols 5, 8, 11
Standard = Col 2
Substrate = Col 3
Blank = Col 1

Oxidase Activity
Activity ($\mu mol\ h^{-1}\ g^{-1}$) = (NAU / L-DOPA Ext. Coeff) × (0.1563 / hours) =
{NAU / [Standard AU / (X µmol/L × 0.00025 L)]} / [0.0002 L × (DW g / 0.125 L) × hours]

NAU = net absorbance units = Assay – (Sample + Substrate)

DW = dry weight of soil sample contained in 125 ml buffer

Assay = Cols 5-6, 8-9, 11-12
Sample = Cols 4, 7, 10
Substrate = Col 2
Blank = Col 1

Calculate the extinction coefficient (in absorbance units/[µmol/L]) by using a purified oxidase enzyme solution (e.g. tyrosinase from Sigma) to oxidize a known amount (50 µM works well) of L-DOPA and measure absorbance of the reaction product after 6 hours. Measure the same volume of L-DOPA product solution into the plates as is used in the assay (250 µl).