Fluorimetric and Oxidative Enzyme Assay Protocol for Microcosms

(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)
4.374 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-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
Acid phosphatase (AP)	4-Methylumbelliferyl phosphate 5.12 mg/100 ml
Polyphenol oxidase (PPO) Peroxidase (PER)	25 mM L-dihydroxyphenylalanine (DOPA) 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 plastic 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 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
Use 50 ml acetate buffer to rinse contents of microcosm vial into a labeled 500 ml polycarbonate canister. Stir with a magnetic stir bar for 1 min, or until the substrate breaks up.

Using a pipet tip with the end cut off, transfer 1 ml of the sample slurry into a labeled 1.5 ml microcentrifuge tube. Freeze at -20ºC.

Assay Set-up
Using 8-channel pipettor and wide-mouth tips (cut off tip ends with scissors), pipette 200 μl of the 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 should be ~30 min for BG, AP, and NAG, and 18-24h for PPO and LAP plates.

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.

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

Fluorescence Activity
Activity (µmol h^{-1} g^{-1}) = (NFU / Standard FU) × (0.625 / hours) =
{NFU / [Standard FU / (10 µmol/L × 0.00005 L)]} / [0.0002 L × (0.2 g / 0.05 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
Blank = Col 1

Oxidase Activity
Activity (μmol h^{-1} g^{-1}) = (NAU / L-DOPA Ext. Coeff) × (0.3125 / hours) =
{NAU / [Standard AU / (X μmol/L × 0.00025 L)]} / [0.0002 L × (0.2 g / 0.05 L) × hours]

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

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

Calculate the extinction coefficient by using a purified oxidase enzyme solution (e.g. tyrosinase from Sigma) to oxidize a known amount of L-DOPA and measure absorbance of the reaction product. Measure the same volume of L-DOPA product solution into the plates as is used in the assay (250 μl).