Soil Enzyme Assays K. DeAngelis October 24, 2005

Materials

N-AcetylGlucosamidine-MUB	Sigma #M2133
Nuclease - Phosphate-MUB	Sigma #3168
MUB (for standards)	Sigma #1381
Phenol Oxidase - L-DihydrOxyPhenylAlanine	Sigma #D9628

O Eppendorf Repeater Multichannel Pipette 4780
O Multi-channel pipettor wells
O Beakers large enough to serve as multi-channel pipettor well
O $NaHCO_3$ for buffer
O Na_2B4O_7 $10H_2O$ for buffer
O 2mm sieved & homogenized soil

Step 1: Prepare reagents and buffers
O Bicarbonate buffer, about 1L per plate (for MUB, phenol oxidase enzyme assays)
Stock sln = 8.4g NaHCO3 in 1L MQ H2O, pH 8.2 = 100mM
Working sln = Dilute 50ml stock in 1L MQ H2O = 5mM
O Prepare protease buffers and reagents as per kit protocol.

Make substrate solutions in appropriate buffer. All solutions store 4°C.

Substrate	reagent	10mM		conc	per plate
N-AG-MUB	-20°C	25 mg	6 ml	100x	50ul in 5 ml
Phosphate-MUB	25°C	25 mg	9.8 ml	100x	50ul in 5 ml
MUB	25°C	25 mg	12.6 ml		
BSA		4mg	1 ml	1x	---
L-DOPA	25°C			1x	0.5 mM

Step 2: Prepare the soil.
O Weigh 1g of each soil and **record weight**, then move into a large 1L beaker.

Step 3:
O Prepare the microplates.

Plate 1-7	beta-glucosamidinase (MUB) = 100 ul MUB-substrate into plates
Plate 8-14	phosphatase (MUB) =100 ul MUB-substrate into plates
Plate 15-21	protease
Plate 22-28	phenol oxidase (L-DOPA) = ..substrate added 2nd ..just label.

O Prepare spectrofluorometer.
Warm up spec for **1 hour**. Fluorolog-3 spectrofluorometer Jobin-Yvon Horiba, lives in the Sposito Lab. Contact Kristen DeAngelis (Firestone lab GS), Andy Yang (Sposito lab manager) &/or Gary Sposito to use this instrument.

O Prepare soil sln

Start with 1g wet soil per treatment and **record weights**. Add 1 g soil to a plastic beaker large enough to fit multichannel pipettor. Add 100 ml 5mM bicarbonate buffer to each. Break up any soil clods. Add stir bar and stir rapidly. Stir until sln looks homogenous (about 1-2 minutes). While sample is being stirred, take 100ul solution with a multi-channel pipettor. You may have to clip off the tips of the pipette tips for easier sampling.

<u>MUB Fluorescence assays</u>

1. Pipette 100 ul of soil sln into appropriate microplate well for all 8 soils, which already have 100 ul appropriate substrate.
2. Place plate into reader. Read it: excitation = 365 nm; emission = 442 nm
3. Place plate into 27C temperature room or incubator, with or without shaking.
4. Read all samples again after 2-4 hours. If sample activity is low (old samples, low organic matter content), you may want to take another measurement at 4 hours. Record times.
5. Calculate nmol of product at beginning and end of incubation based on quenched standards. I have found that standards change fluorescence over time so you have to calculate nmol of product based upon standards read at the same time. Also remember that you are diluting the standards 2X in this assay.
6. Calculate activity as nmol substrate converted per hour per ml of extract.

   Actual (nmol h-1 g soil-1) = (mean final nmol- mean initial nmol)/ (dilution factor * assay interval (hr)))

<u>Protease assay</u>

<u>Phenol oxidase assay:</u>

1. Use clear, flat-bottom 96-well plates. Place 100 ul of the soil sln into each well.
2. While the spectrofluorometer plates are incubating, add 100ul of 5mM L-DOPA to rows 1-8 (soil) and only buffer as controls.
3. Incubate these samples at 27C and measure the increase in absorbance at 460nm every 5 minutes for 1 hr.

	1	2	3	4	5	6	7	8	9	10	Stds 10, 11
A	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	200 B
B	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 B + 8 MUB + 92 H20
C	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 B + 20 MUB + 80 H20
D	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 B + 40 MUB + 60 H20
E	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 B + 60 MUB + 40 H20
F	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 B + 80 MUB + 20 H20
G	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 B + 100 MUB
H	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	100 sub 100 soil	**100 B** **100 soil**	

Plate 1-7 beta-glucosamidinase (MUB) sub = beta-glucosaminidase-MUB

Standards 1 2 3 4 5 6 7

O B = buffer

O MUB

O Water

Experiments

O B = buffer

O sub = Substrate MUB

O Soil

Plate 8-14 phosphatase (MUB) sub = phosphate-MUB

Standards 8 9 10 11 12 13 14

O B = buffer

O MUB

O Water

Experiments

O B = buffer

O sub = Substrate MUB

O Soil

Plate 15-21 protease

	Treatment 1	2	3	4	5	6	7	8	9	10	11
A	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
B	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
C	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
D	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
E	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
F	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
G	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B
H	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	50ul (1.2) 100ul soil 50ul (1.3)	**100ul (1.2)** **100ul soil**	100ul (1.2) 100ul B

15 16 17 18 19 20 21

O (1.2) 2x digestion buffer

O soil

O B = bicarbonate buffer

O (1.3) BODIPY casein substrate

Plate 22-28 phenol oxidase (L-DOPA) sub = L-DOPA

	1	2	3	4	5	6	7	8	9	10	11
A	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
B	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
C	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
D	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
E	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
F	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
G	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only
H	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	100ul sub 100ul soil	**100ul B** **100ul soil**	B only

22 23 24 25 26 27 28

O B = buffer

O sub = Substrate L- DOPA

O Soil

	1	2	3	4	5	6	7	8	9	10	Stds 11,12
A	R1 – S0	R1 – S0	R1 – S0	R1 – S0	B	R3 – S0	R3 – S0	R3 – S0	R3 – S0	B	B
B	R1 – S1	R1 – S1	R1 – S1	R1 – S1	B	R3 – S1	R3 – S1	R3 – S1	R3 – S1	B	10
C	R1 – S2	R1 – S2	R1 – S2	R1 – S2	B	R3 – S2	R3 – S2	R3 – S2	R3 – S2	B	20
D	R1 – S3	R1 – S3	R1 – S3	R1 – S3	B	R3 – S3	R3 – S3	R3 – S3	R3 – S3	B	40
E	R2 – S0	R2 – S0	R2 – S0	R2 – S0	B	R4 – S0	R4 – S0	R4 – S0	R4 – S0	B	60
F	R2 – S1	R2 – S1	R2 – S1	R2 – S1	B	R4 – S1	R4 – S1	R4 – S1	R4 – S1	B	80
G	R2 – S2	R2 – S2	R2 – S2	R2 – S2	B	R4 – S2	R4 – S2	R4 – S2	R4 – S2	B	100
H	R2 – S3	R2 – S3	R2 – S3	R2 – S3	B	R4 – S3	R4 – S3	R4 – S3	R4 – S3	B	

	PLATE		Treatm' t	Rep ucsm	Soil Type		PLATE		Treatm' t	Rep ucsm	Soil Type
1	1,8,15,22	A 1-4	**1**	**1**	0 – bulk	57	4, 11,18,25	**A 6-9**	**4**	**3**	0 – bulk
2	1,8,15,22	B 1-4		-	1 – tip	58	4, 11,18,25	**B 6-9**		-	1 – tip
3	1,8,15,22	C 1-4		-	2 – hairs	59	4, 11,18,25	**C 6-9**		-	2 – hairs
4	1,8,15,22	D 1-4		-	3 - mature	60	4, 11,18,25	**D 6-9**		-	3 - mature
5	1,8,15,22	E 1-4		**2**	0 – bulk	61	4, 11,18,25	**E 6-9**		**4**	0 – bulk
6	1,8,15,22	F 1-4		-	1 – tip	62	4, 11,18,25	**F 6-9**		-	1 – tip
7	1,8,15,22	G 1-4		-	2 – hairs	63	4, 11,18,25	**G 6-9**		-	2 – hairs
8	1,8,15,22	H 1-4		-	3 - mature	64	4, 11,18,25	**H 6-9**		-	3 - mature
9	1,8,15,22	**A 6-9**		**3**	0 – bulk	**65**	5, 12,18,26	A 1-4	**5**	**1**	0 – bulk
10	1,8,15,22	**B 6-9**		-	1 – tip	**66**	5, 12,18,26	B 1-4		-	1 – tip
11	1,8,15,22	**C 6-9**		-	2 – hairs	**67**	5, 12,18,26	C 1-4		-	2 – hairs
12	1,8,15,22	**D 6-9**		-	3 - mature	**68**	5, 12,18,26	D 1-4		-	3 - mature
13	1,8,15,22	**E 6-9**		**4**	0 – bulk	**69**	5, 12,18,26	E 1-4		**2**	0 – bulk
14	1,8,15,22	**F 6-9**		-	1 – tip	**70**	5, 12,18,26	F 1-4		-	1 – tip
15	1,8,15,22	**G 6-9**		-	2 – hairs	**71**	5, 12,18,26	G 1-4		-	2 – hairs
16	1,8,15,22	**H 6-9**		-	3 - mature	**72**	5, 12,18,26	H 1-4		-	3 - mature
17	2, 9, 16, 23	A 1-4	**2**	**1**	0 – bulk	**73**	5, 12,18,26	**A 6-9**		**3**	0 – bulk
18	2, 9, 16, 23	B 1-4		-	1 – tip	**74**	5, 12,18,26	**B 6-9**		-	1 – tip
19	2, 9, 16, 23	C 1-4		-	2 – hairs	**75**	5, 12,18,26	**C 6-9**		-	2 – hairs
20	2, 9, 16, 23	D 1-4		-	3 - mature	**76**	5, 12,18,26	**D 6-9**		-	3 - mature
21	2, 9, 16, 23	E 1-4		**2**	0 – bulk	**77**	5, 12,18,26	**E 6-9**		**4**	0 – bulk
22	2, 9, 16, 23	F 1-4		-	1 – tip	**78**	5, 12,18,26	**F 6-9**		-	1 – tip
23	2, 9, 16, 23	G 1-4		-	2 – hairs	**79**	5, 12,18,26	**G 6-9**		-	2 – hairs
24	2, 9, 16, 23	H 1-4		-	3 - mature	**80**	5, 12,18,26	**H 6-9**		-	3 - mature
25	2, 9, 16, 23	**A 6-9**		**3**	0 – bulk	81	6, 13,19,27	A 1-4	**6**	**1**	0 – bulk
26	2, 9, 16, 23	**B 6-9**		-	1 – tip	82	6, 13,19,27	B 1-4		-	1 – tip
27	2, 9, 16, 23	**C 6-9**		-	2 – hairs	83	6, 13,19,27	C 1-4		-	2 – hairs
28	2, 9, 16, 23	**D 6-9**		-	3 - mature	84	6, 13,19,27	D 1-4		-	3 - mature
29	2, 9, 16, 23	**E 6-9**		**4**	0 – bulk	85	6, 13,19,27	E 1-4		**2**	0 – bulk
30	2, 9, 16, 23	**F 6-9**		-	1 – tip	86	6, 13,19,27	F 1-4		-	1 – tip
31	2, 9, 16, 23	**G 6-9**		-	2 – hairs	87	6, 13,19,27	G 1-4		-	2 – hairs
32	2, 9, 16, 23	**H 6-9**		-	3 - mature	88	6, 13,19,27	H 1-4		-	3 - mature
33	3,10,17,24	A 1-4	**3**	**1**	0 – bulk	89	6, 13,19,27	**A 6-9**		**3**	0 – bulk
34	3,10,17,24	B 1-4		-	1 – tip	90	6, 13,19,27	**B 6-9**		-	1 – tip
35	3,10,17,24	C 1-4		-	2 – hairs	91	6, 13,19,27	**C 6-9**		-	2 – hairs
36	3,10,17,24	D 1-4		-	3 - mature	92	6, 13,19,27	**D 6-9**		-	3 - mature
37	3,10,17,24	E 1-4		**2**	0 – bulk	93	6, 13,19,27	**E 6-9**		**4**	0 – bulk
38	3,10,17,24	F 1-4		-	1 – tip	94	6, 13,19,27	**F 6-9**		-	1 – tip
39	3,10,17,24	G 1-4		-	2 – hairs	95	6, 13,19,27	**G 6-9**		-	2 – hairs
40	3,10,17,24	H 1-4		-	3 - mature	96	6, 13,19,27	**H 6-9**		-	3 - mature
41	3,10,17,24	**A 6-9**		**3**	0 – bulk	**97**	7, 14,20,28	A 1-4	**7**	**1**	0 – bulk
42	3,10,17,24	**B 6-9**		-	1 – tip	**98**	7, 14,20,28	B 1-4		-	1 – tip
43	3,10,17,24	**C 6-9**		-	2 – hairs	**99**	7, 14,20,28	C 1-4		-	2 – hairs
44	3,10,17,24	**D 6-9**		-	3 - mature	**100**	7, 14,20,28	D 1-4		-	3 - mature
45	3,10,17,24	**E 6-9**		**4**	0 – bulk	**101**	7, 14,20,28	E 1-4		**2**	0 – bulk
46	3,10,17,24	**F 6-9**		-	1 – tip	**102**	7, 14,20,28	F 1-4		-	1 – tip
47	3,10,17,24	**G 6-9**		-	2 – hairs	**103**	7, 14,20,28	G 1-4		-	2 – hairs
48	3,10,17,24	**H 6-9**		-	3 - mature	**104**	7, 14,20,28	H 1-4		-	3 - mature
49	4, 11,18,25	A 1-4	**4**	**1**	0 – bulk	**105**	7, 14,20,28	**A 6-9**		**3**	0 – bulk
50	4, 11,18,25	B 1-4		-	1 – tip	**106**	7, 14,20,28	**B 6-9**		-	1 – tip
51	4, 11,18,25	C 1-4		-	2 – hairs	**107**	7, 14,20,28	**C 6-9**		-	2 – hairs
52	4, 11,18,25	D 1-4		-	3 - mature	**108**	7, 14,20,28	**D 6-9**		-	3 - mature
53	4, 11,18,25	E 1-4		**2**	0 – bulk	**109**	7, 14,20,28	**E 6-9**		**4**	0 – bulk
54	4, 11,18,25	F 1-4		-	1 – tip	**110**	7, 14,20,28	**F 6-9**		-	1 – tip
55	4, 11,18,25	G 1-4		-	2 – hairs	**111**	7, 14,20,28	**G 6-9**		-	2 – hairs
56	4, 11,18,25	H 1-4		-	3 - mature	**112**	7, 14,20,28	**H 6-9**		-	3 - mature