

Suding Lab Enzyme Assay Protocol

(Modified from S. Schmidt Lab/M. Weintraub U. of Colorado Boulder, August 2005)

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Materials needed:

Item	Catalog #
4-methylumbelliferyl B-D-xylopyranoside	Sigma M7008 100 mg
L-Leucine-7-amido-4-methylcoumarin hydrochloride	Sigma L2145 25 mg
4-Methylumbelliferyl β -D-glucopyranoside	Sigma M3633 250 mg
4-Methylumbelliferyl N-acetyl- β -D-glucosaminide	Sigma M2133 100 mg
4-Methylumbelliferyl β -D-cellobioside	Sigma M6018 100 mg
7-Amino-4-methylcoumarin	Sigma A9891 250 mg
4-Methylumbelliferyl α -D-glucopyranoside	Sigma M9766 100 mg
4-Methylumbelliferyl phosphate	Sigma M8883 250 mg
N-acetyl-D-Glucosamine	A8625 25 g
L-Dopa 99%	167530050 5 g
8-Channel Pipetter, 5-50 microliters	21-377-246
8-Channel Pipetter, 50-300 microliters	21-377-247
Fisherbrand Redi-Tip Pipet Tips hinged rack 5 x 96	02-707-506
Finntip 02.-50 microliter pipette tips rack 1 x 384	21-377-294
Disposable pipetting reservoir 50 pack, 100 mL capacity	13-712-14
Fisherbrand* Octagonal Magnetic Stir Bar Kit	14-513-82
Fisher Lab Disc Magnetic Stirrers	14-505-122
Wheaton Leak-Resistant Narrow-Mouth Natural LDPE Bottles 100 mL pack of 12	03-083-5
Costar* 96-Well Black Well Plates	07-200-590
Fisherbrand* Clear Polystyrene 96-Well Plates	12-565-501
LABCHEM INC SODIUM ACETATE 1M 1L	LC07313
Nalgene* Transparent Polymethylpentene Jars 16. oz pack of 4	11-823-32
BioSpec Biomixer* Blender	11-504-204
BioTek Plate Reader	
Incubator	

I. Pre-prep

1. Make substrate and fluorescent label control solutions in 125mL plastic bottles. Store solutions in the 4°C refrigerator. Substrates are in freezer. Remake solutions~every 2 weeks or as needed. Recipes:

β -D-cellobiosidase (CBH)	10 mg/100 mL dionized (DI) water
α -Glucosidase (AG)	6.77mg/100 mL
β -Glucosidase (BG)	6.77mg/100 mL
L-Leucine 7-amidomethylcoumarin (LAP)	6.5 mg/100 mL
N-acetyl- β -glucosaminidase (NAG)	7.59 mg/100 mL
4 MUB Phosphatase (PHOS)	5.12 mg/100 mL
β -Xylosidase (BXYL)	6.17 mg/100 mL
MUB	1.76 mg/100 mL
MC	1.75 mg/100 mL

2. Prepare 2L 50mM working solution of sodium acetate buffer by mixing 50ml 1M sodium acetate, pH 5.0 stock solution with 950mL DI H₂O. Calibrate pH meter, measure pH of solution. Add ~15 drops of glacial acetic acid to 1L bottle, mix well, measure pH. If pH is above 5.0, add 1 drop at a time, mix, measure until the solution reaches pH 5.0.

3. Prepare fresh solution of 0.3% H₂O₂ (25 mL or 50 mL as needed) using DI water. Place in labeled 125ml bottle. Store at 4°C. Recipe: 2.5mL H₂O₂/22.5mL DI water; or 5mL H₂O₂/45mL DI water.

4. Prepare fresh solution of L-DOPA using DI water. Heat the water first in the microwave for 1 min. Shake well. If necessary, place the solution on a stir plate to ensure all the solid is dissolved. 25-50 mL as needed. Recipe: 0.247g/50mL; 0.124/25mL.

5. Label the black and clear 96 well plates with tape.

- For each soil sample, 9 substrates are used. Each plate holds three samples.
- Label seven fluor (black) plates with the substrate names: LAP, AG, BG, BXYL, Phos, CBH, & NAG
- Label the spec (clear) plates with the substrate names: Phenox, Perox,
- Label one fluor plate as "Soil + Buffer" for each 12 samples.

For an assay with more than three soil samples, multiple plates for each substrate will be necessary (LAP 1, LAP 2, LAP 3, etc.). 3 samples can be run per plate, so running 12 samples, for example, will require 4 plates for each assay – 28 black plates, and 8 clear plates, total.

II. Slurry Prep

1. Weigh one gram of soil sample into a wide-mouth plastic container. Soil sample should be frozen and care should be taken to prevent thawing while weighing out the sample (Weigh one samples at a time, place back in freezer, or use a cooler).
2. Add 125 mL 50 mM sodium acetate buffer, pH 5.0
3. Blend contents with handheld blender (high) for 1 minute (use a timer!)
4. Keep soil slurry refrigerated until used.
5. Rinse off blender with buffer between samples.

III. Pipetting Samples

1. Using multichannel pipettors and wide-mouth tips, pipette 200µl of the soil slurry into the 96 well plates (except the "Soil + Buffer" plate), keeping slurry well stirred with a stir bar/plate, as indicated. Where 1, 2, 3 are the different soil samples. Note on datasheet which sample is which. All assay plates should be pipetted as above, leaving columns 1-3 empty.

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

A	none	none	none	1	1	1	2	2	2	3	3	3
B	none	none	none	1	1	1	2	2	2	3	3	3
C	none	none	none	1	1	1	2	2	2	3	3	3
D	none	none	none	1	1	1	2	2	2	3	3	3
E	none	none	none	1	1	1	2	2	2	3	3	3
F	none	none	none	1	1	1	2	2	2	3	3	3
G	none	none	none	1	1	1	2	2	2	3	3	3
H	none	none	none	1	1	1	2	2	2	3	3	3

2. Pipette one column of each sample into the “Soil + Buffer” plate with sample one in column one, sample two in column two, etc. Multiple plates should be pipetted the same way.

IV. Pipetting Buffer

Pipette 50mM Na Acetate, pH 5.0 according to the following chart:

	200 uL of buffer	50 uL of buffer
Fluor (black) Plates	Columns 1, 2, 3	Column 1
Spec (clear) Plates	Columns 1, 2	Columns 1, 4, 7, 10

V. Pipetting Fluorescent Standards and Substrate (Fluor Plates Only)

- For all plates except the LAP plates:
Pipette 50 uL of MUB into columns 2, 4, 7, 10
- For LAP plates
Pipette 50 uL of MC into columns 2, 4, 7, 10
- Pipette 50 uL of substrate into their respective plates, noting time
Columns 3, 5, 6, 8, 9, 11, 12

VI. Pipetting Substrates (Spec Plates only)

- Add substrates.
 - Phenox Plates
Add 50 uL of L-DOPA, noting time
Columns 2, 5, 6, 8, 9, 11, 12
 - Perox Plates
 - Add 50 uL of L-DOPA
Columns 2, 5, 6, 8, 9, 11, 12
 - Add 10 uL of H₂O₂ to all columns (except 2), noting time
- Let plates sit overnight in a 13° C incubator.

	Fluorescent Plates	Spectrophotometric Plates
1	250 uL buffer	250 uL buffer

2	200 uL buffer + 50 uL fluor label	200 uL buffer + 50 uL substrate
3	200 uL buffer + 50 uL substrate	Empty
4	200 uL sample 1 + 50 uL fluor label	200 uL sample 1 + 50 uL buffer
5	200 uL sample 1 + 50 uL substrate	200 uL sample 1 + 50 uL substrate
6	200 uL sample 1 + 50 uL substrate	200 uL sample 1 + 50 uL substrate
7	200 uL sample 2 + 50 uL fluor label	200 uL sample 2 + 50 uL buffer
8	200 uL sample 2 + 50 uL substrate	200 uL sample 2 + 50 uL substrate
9	200 uL sample 2 + 50 uL substrate	200 uL sample 2 + 50 uL substrate
10	200 uL sample 3 + 50 uL fluor label	200 uL sample 3 + 50 uL buffer
11	200 uL sample 3 + 50 uL substrate	200 uL sample 3 + 50 uL substrate
12	200 uL sample 3 + 50 uL substrate	200 uL sample 3 + 50 uL substrate

VII. Pre Reading Prep

A. Turn on Fluorescence Plate Reader, computer, MSExcel, and KC4 software.

B. Add 10 uL of 0.5M NaOH to each well of all fluorescence 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.)

C. Wipe condensation from plate bottoms using a paper towel before putting into the plate reader. This is especially important for the clear plates. The data for these plates will not be accurate unless you wipe the plate bottoms off first.

VIII. Plate readings

A. Fluorescence plates

1. Open KC4 and Excel.
2. In KC4, check settings.

System menu→Readers→Filters

For setup make sure Emission filter A is 460nm, bandwidth 40. Absorbance is NOT checked, change if needed. Choose “OK”, “Close”.

3. Set protocol: Protocol menu→Open “Enzyme”.

Check settings. Choose settings (picture menu bar).

Filter set 1/Fluorescence.

Excitation=360/40

Emission=460/40

Optics=Top

Sensitivity=75

Plate type=96 well

“OVFLW” readings means sensitivity is too high, change as necessary (but change for soil+buffer controls too.)

4. Read plate. Read→Start Reading→OK. At prompt, insert the plate.
5. When results come up, save plate in Excel. Use mouse to select plate, copy and paste into Excel. Save Excel file with date, each worksheet is different enzyme.
6. Close old plate, read new.

Choose New, “no” to save changes. Read the next plate.

B. Spec Plates (Phenox/Perox)

1. Check settings in KC4.

System menu→Readers→Filters

For setup make sure Emission filter A- check Absorbance box. Choose “OK”, “Close”.

2. Set protocol: Protocol menu→Open “Enzyme Absorbance”

Filter set 1= 460.

Check Absorbance

Optics=Bottom

Plate type=96 well

3. Read plate, save data as above.