Enzyme Assay Protocol

Required Glassware/Equipment:

1 x Calibrated 125 mL Erlenmeyer flask
2 x 50 mL beaker
2 x 125 mL Nalgene bottle
Pre-weighed (~1.00 g) soil samples
6 x black 96-well plates (per soil set)
2 x clear 96-well plates (per soil set)
Pipette bulb and 25 mL Falcon pipette
Homogenizer (Tissue Tearor)
Multichannel and single channel pipettes (5-50 μL, 20-200 μL and 200-1200 μL)
Pipette Tips (Rainin P200 & P1000)
Microwave, scale and plate reader

Safety and Sterile Technique

-Before setting up for enzymes the counter should be wiped down with 70% ethanol

-Anytime that you are touching the enzyme plates you should wear gloves that have been washed clean and cleaned with ethanol

-When using the homogenizer, be sure to leave the stainless steel tip in the soil slurry solution until all rotation has stopped. Failure to do so may result in contamination of nearby plates.

-Wear gloves while pipetting in the lab and when adding 0.5 M sodium hydroxide to the enzyme assay plates

-Be sure to wear gloves while handling plates during reading, but do NOT touch computer with gloves. If you are using the 4th floor plate reader, ALWAYS where gloves.

-If any chemicals used for enzyme analysis come in contact with your skin:
>Rinse the affected area for 15 minutes under water
>Notify your supervisor

-If any chemicals used for enzyme analysis come in contact with your eyes0:
>Rinse your eyes for 15 minutes at the eye wash station located at the east sink in the laboratory
>Notify your supervisor

Reagents:

0.5 M NaOH
2.00 g NaOH Pellets
100 mL DI water

50 mM sodium acetate buffer
4.10 g anhydrous sodium acetate
1 L DI water
Titrate with glacial acetic to pH ~6

Substrate Solutions (200 μM unless otherwise noted):

Enzyme	Substrate	Note	Plate Incubation Time
β-Glucosidase (BG)	4-Methyllumbelliferyl β-D-glucopyranoside 6.77 mg/100 mL	Stored in the -20 °C	1.5 – 2.5 hours
β-Xylosidase (BX)	4-Methylumbelliferyl β-D-xylospyranoside 6.17 mg/100 mL	Stored in the -20 °C	1.5 – 2.5 hours
Cellobiohyrolase (CB)	4-Methylumbelliferyl β-D-cellobioside 10.01 mg/100 mL	Stored in the 4 °C	1.5 – 2.5 hours
β-N-acetylglucosaminidase (NAG)	4-Methylumbelliferyl N-acetyl-β-D-glucosaminide 7.59 mg/100 mL	Stored in the -20 °C	0.5 hours
Leucine aminopeptidase (LAP)	L-Leucine-7-amido-4-methylcoumarin hydrochloride 6.5 mg/100 mL	Stored in the -20 °C	18 – 24 hours
Acid Phosphatase (AP)	4-Methylumbelliferyl phosphate 5.12 mg/100 mL	Stored in the -20 °C	0.5 hours
Polyphenol Oxidase (PPO)	25 mM L-dihydroxyphenylalanine (DOPA) 123.25 mg/25 mL (makes enough for 4 plates) 246.5 mg/50 mL (makes enough for 8 plates)	Make fresh in the morning-long stir time	18 – 24 hours
Peroxidase (PER)	0.30 % Hydrogen peroxide 2 mL 3% certified H_2O_2- bring up to 20 mL with DI water	Make fresh in the morning	18 – 24 hours
MUB standard	100 μM 4-Methylumbelliferone 0.88 mg/ 500 mL	Make fresh every 2 weeks	N/A
AMC standard	100 μM 7-Amino-4-methylcoumarin	Stored in the -20 °C	N/A

***Follow the diagram when adding reagents to the plates. The LAP enzyme test uses LAP as the substrate and AMC as the "MUB"**

Preparation

Substrates:
Good for up to two months if stored in the -20 °C freezer.
Prepare substrate solutions in amber bottles, aliquot (12-13 mL) into 15 mL centrifuge tubes and store in the -20 °C freezer.

NOTE: L-DOPA and 0.3% hydrogen peroxide solution must be made fresh on the day of analysis. "Recipes" on previous page. Sodium Acetate Buffer viable for 1 month.

96-Well Plates:
Black 96-well plates are used for substrates corresponding with the following enzymes: BG, NAG, BX, AP, CB and LAP. Clear 96-well plates are used for phenol oxidase and peroxidase plates. The plate reader should be turned on and allowed ample time to warm up before taking readings.

Label the 96-well plates based on the enzyme being tested and set number. For example, if you were running the first set you would label the plates BG 1, NAG 1, BX 1, AP 1, CB 1, PO 1, PER 1 & LAP 1.

Homogenizing:

-Fill Erlenmeyer flask with 125ml of Sodium Acetate buffer
-Pour half into 125ml Nalgene bottle with ~1.00 g soil sample (record actual soil weight)
-Homogenize for 30 seconds
-Pour the remainder of 125ml sodium acetate buffer into bottle and homogenize for another 10 seconds
-Clean homogenizer—place in first sodium acetate buffer rinse for 10 seconds and then in second sodium acetate buffer rinse for 10 seconds.

Plate Setup:

Fluorometric Assays

	1 Blank	2 Ref.	3 NegCon	4 Quench	5 Cont.	6 Assay	7 Quench	8 Cont.	9 Assay	10 Quench	11 Cont.	12 Assay
200 µl	buffer	buffer	buffer	soil 1	soil 1	soil 1	soil 2	soil 2	soil 2	soil 3	soil 3	soil 3
50 µl	buffer	MUB	sub	MUB	buffer	sub	MUB	buffer	sub	MUB	buffer	sub
A												
B												
C												
D												
E												
F												
G												
H												

Reagents:
50 mM acetate buffer, pH=5
200 µM substrate
10 µM standard (MUB)
10 µl 0.5 N NaOH

Read Fluorescence (Wavelengths: Excitation = 365nm & Emission = 450nm)

JL DeForest
OHIO University

When setting up plates, the illustration above is used. The diagram is set up to show what solutions need to go in certain wells on a 96-well plate. This particular diagram is for a fluorometric enzyme assay and therefore would be used on a black plate when testing for BG, NAG, BX, AP, CB & LAP enzyme activity. The numbers 1-12 represent the columns of wells that are found on a 96-well plate and the letters A-H represent the rows of wells found on a plate. When using this chart it is important to understand that the colored solutions at the top of each column represent the amount of each solution that will go into every well of that column.

For example, when setting up a black 96-well plate you would pipette (using a multichannel pipette) 200 μL of buffer into wells 1A-1H, 2A-2H and 3A-3H. Next, using clean pipette tips, you would pipette 200 μL of soil slurry 1 into wells 4A-4H, 5A-5H and 6A-6H. You would then repeat this process for soil samples 2 and 3. Following the 200 μL additions, you would then continue by adding 50 μL of the specified solutions to their corresponding wells.

Creating a Protocol in Gen5:
Note: Fluorometric and absorbance protocols have already been created for Hofmockel Laboratory use and can be accessed by following steps 15 & 16 in the procedure below.

To create a new protocol in the Gen5 program, select "protocol" under the "create new" box. A blank screen will appear with a list of icons on the left side of the screen. Select the first icon (protocol). A box will now open with buttons on the left side. While building your protocol, you will select each of these buttons for each step of the protocol. You must determine these settings in chronological order. When creating the protocol it is important to note that fluorescence excitation is measured at 365 nm and emission is measured at 450 nm.

When the protocol is complete click OK and you will return to a blank screen. Select "plate map" and a new box will open which displays the layout of a blank 96-well plate. Using the options from the drop-down menu, specify what each of the wells on the plate will contain (sample, control, blank, etc.) and click OK upon completion of this step.

To specify how the data is exported into an Excel file you must double-click "protocol options" and select "quick export settings."

Enzyme Assay Procedure

1) Clean your work area with 70% ethanol and Kimwipes/paper towels

2) If substrate solutions have already been made, aliquoted and frozen, place them in the hot water bath (they will need about 10 minutes to thaw) and continue on to step six. If the substrate solutions have not already been prepared then continue onto the next step.

3) Using the recipes found on page two and the microbalance found in Science I, weigh out the appropriate amount of solid substrate and place into an appropriate mixing container (125 mL amber bottles for most substrates).

4) Add the appropriate amount of deionized water (gravimetrically unless using a volumetric flask).

5) Place the solution on a stir plate with a stir bar added and stir at about 1000 rpm until all solid has gone into solution.

6) After all other substrate solutions have begun to thaw (for frozen) or mix (for fresh), L-DOPA and hydrogen peroxide solutions must be made. These solutions should be made fresh each day that the enzyme assay is set up and should be made following the recipes on page two. Both L-DOPA and

hydrogen peroxide are light sensitive; therefore the solutions should be made in a low light environment and stored in a covered container.

7) Label clean 96-well plates and place them in the order that you would like to pipette them in. You will need six black plates and two clear plates for each set of soils that you run.

8) Label 11 clean boats (BG, NAG, BX, AP, CB, PO, PER, LAP, AMC, MUB and Buffer)

9) After preparing for plate setup the plate reader and Gen5 program should now be turned on to allow ample time for warm-up. A small black switch on the lower-left hand corner of the front of the plate reader must be switched on to turn on the plate reader. Following the warm-up period, the Gen00005 icon on the computer's desktop must be double-clicked.

11) When all solutions have finished mixing/thawing they can be poured into their corresponding boat.

12) Next, homogenize the three soil samples that you will be using for the enzyme assay. Use the directions found on page three of this protocol and place the soils into the plastic Nalgene tubs in an ascending numerical order. The soil slurry solutions should have a large stir bar in them and the stir plates should be set at about 350 rpm with the temperature turned off.

13) Following the directions in the "Plate Setup" section on pages three and four, set up all eight enzyme assay plates. Be sure to record the time of substrate addition and determine at what time you will be reading the plates on the plate reader (This can be done by using the given incubation time on page two for each enzyme being tested).

14) Immediately following the plate setup, place all plates into the incubation drawer (dark and ~20 °C). Incubation times vary by enzyme- see the table on page two to determine the correct incubation time for each plate.

15) When a plate has incubated for five minutes less than its full incubation time it should be taken to the plate reader along with the other supplies necessary for reading plates. These supplies include: the 5-50 μL multichannel Rainin pipette, a box of P200 Rainin pipette tips (green box), a pair of gloves, 0.5 M NaOH with a boat and the enzyme plates that you will be reading.

16) If reading absorbance (for PO and PER) open a new experiment on the Gen5 menu and select the appropriate protocol. Place your plate in the plate reader and click the start reading button. Click OK on the popup and record the reading start time for the plate. Continue on step 18.

17) If reading fluorescence (for BG, NAG, BX, AP, CB or LAP) open a new experiment on the Gen5 menu and select the appropriate protocol. Place your plate in the plate reader and proceed to step 17.

NOTE: The first time that a fluorometric plate is read it will require a three minute (180 second) wait for bulb warm-up.

18) 10 μL of 0.5 M NaOH is added to all 96 wells for florescence readings to increase the fluorescence and decrease quench values. Before reading, add the NaOH to all wells using a Rainin multichannel pipette. Immediately set a timer for one minute. During this time you should place the plate onto the reader tray and click start reading so you can start the reading immediately following the one-minute delay by pushing OK in the popup. Record the reading start time for the plate.

19) After all 96 wells have been read, 96 values will appear in a diagram setup like a plate. Maximize your Excel spreadsheet and select the appropriate cells in your Excel data collection spreadsheet. Return to your results in Gen5 and click the data export button. Be sure to correctly label your data in your spreadsheet and include the date and time that the plate was read.

20) After the results have been displayed in Gen5 you should save them directly in the program. Click the SAVE icon after each plate has been read to prevent data loss.

Data Analysis

Enzyme activities were expressed in units of nmol h^{-1} g^{-1} and calculated by the following equations:

$$\text{Activity (nmol } h^{-1} g^{-1}) = \frac{\text{net fluor.} * 125\,\text{ml}}{\text{emission coef.} * 0.2\ \text{ml} * \text{time (hr)} * \text{soil (g)}}$$

Where:

$$\text{Net fluor.} = \left(\frac{\text{sample assay - soil control}}{\text{quench coefficient}} \right) - \text{negative control}$$

$$\text{Emission coef. (fluor nmole}^{-1}) = \frac{\text{reference standard}}{0.5\ \text{nmol}}$$

$$\text{Quench coef.} = \frac{\text{(quench standard - soil control)}}{\text{reference standard}}$$

What to Expect: Typical Data Results for Enzyme Assays

Fluorometric Assays

	1 Blank	2 Ref.	3 NegCon	4 Quench	5 Cont.	6 Assay	7 Quench	8 Cont.	9 Assay	10 Quench	11 Cont.	12 Assay
200 µl	buffer	buffer	buffer	soil 1	soil 1	soil 1	soil 2	soil 2	soil 2	soil 3	soil 3	soil 3
50 µl	buffer	MUB	sub	MUB	buffer	sub	MUB	buffer	sub	MUB	buffer	sub
A												
B												
C												
D												
E												
F												
G												
H												

Reagents:

50 mM acetate buffer, pH=5
200 µM substrate
10 µM standard (MUB)
10 µl 0.5 N NaOH

Read Fluorescence (Wavelengths: Excitation = 365nm & Emission = 450nm)

JL DeForest
OHIO University

NAG

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	11427	77	7269	31	660	10113	39	793	6756	24	498
B	18	9456	83	6581	37	591	11685	39	583	8342	28	358
C	16	10074	96	5477	43	838	9292	42	1097	5370	32	581
D	34	12661	93	7542	41	580	9173	45	660	9220	33	513
E	15	16041	70	6255	41	617	11242	45	610	9362	31	769
F	23	16477	87	7563	42	608	9277	50	714	6409	34	526
G	24	14472	89	8295	42	514	9183	51	723	8858	38	639
H	19	15330	100	9050	42	597	9327	54	870	8744	33	428

AP

	1	2	3	4	5	6	7	8	9	10	11	12
A	12	12061	958	11074	35	1621	4877	49	5764	5276	33	4528
B	19	14762	962	10617	43	1493	4790	48	4852	3673	32	4121
C	16	14952	1183	10621	44	1602	6733	50	6533	5361	33	4721
D	15	11932	959	10749	43	1588	9365	54	7186	6407	41	4638
E	13	17989	974	10998	41	1444	7775	56	5667	5060	37	6518
F	17	17589	899	11047	46	1707	10065	54	4360	9531	45	5440
G	16	10382	1028	10793	71	1768	8513	55	7289	9667	42	5893
H	15	15554	1001	11155	47	1360	8635	66	3759	5808	40	4187

BG

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	8538	47	7934	43	4359	9863	47	7831	8565	41	7237
B	10	13833	57	9075	51	6032	9236	52	11416	9403	42	7003
C	29	12919	52	9560	46	7370	8075	59	11280	9519	47	10603
D	6	15832	59	9051	50	5431	9781	57	11694	10052	43	4900
E	7	13089	61	10122	52	7210	7403	60	11380	8518	44	9180
F	12	11282	66	10070	55	10372	8391	59	6287	9166	48	8390
G	9	13524	63	10227	57	7967	11109	60	11299	9443	50	6828
H	7	12393	63	7799	53	6649	10818	62	10889	9684	52	8802

BX

	1	2	3	4	5	6	7	8	9	10	11	12
A	6	12767	116	9707	56	619	11116	88	1272	10321	62	745
B	10	16403	103	6797	59	899	13180	85	1182	10580	63	1105
C	9	16409	123	5760	59	617	12497	87	1286	10690	61	486
D	9	16282	94	6912	69	1046	11856	103	1272	8431	70	683
E	11	15915	111	10648	67	706	12941	90	1155	11291	65	970
F	12	11543	105	9181	65	756	11186	81	1033	11198	69	935
G	16	16358	100	10688	67	572	12342	83	1248	7876	66	435
H	11	14468	120	9933	65	433	13206	93	1319	11527	72	988

CB

	1	2	3	4	5	6	7	8	9	10	11	12
A	6	12329	104	9028	47	2949	10010	51	2426	10827	51	2139
B	11	14340	137	9374	53	2540	8763	54	2349	7507	49	2410
C	12	13549	116	9347	54	2350	10701	55	3556	10160	57	3035
D	13	15388	156	9661	55	2128	11253	57	2734	8243	54	1952
E	11	15754	134	8451	56	2696	11096	61	2683	10709	60	3403
F	13	13853	175	9346	57	2829	12020	63	2233	10865	61	2159
G	16	16236	160	8187	59	3187	11749	65	2807	8334	60	2725
H	13	11743	141	6639	57	2320	11986	70	4013	11363	68	2535

LAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	7	5241	3241	2501	54	8603	2595	50	8294	2369	48	7646
B	10	4682	3655	2401	54	7094	2629	55	8725	2037	55	6872
C	11	4819	3687	2627	61	9394	2659	60	8779	1933	59	7736
D	10	4635	3659	2307	62	8163	2680	58	10451	2137	58	8485
E	10	4816	3926	2364	66	9368	2857	58	9986	2043	63	8045
F	11	4675	3833	2313	70	9228	2754	64	11409	2013	78	8608
G	13	4309	3759	2352	63	8758	2841	70	7537	2271	51	8088
H	11	3855	3775	2164	65	7132	2124	61	7345	1973	54	6877