Microplate Enzyme Assay Using Fluorescence

Original Reference: [Include a citable reference even if you got a protocol from another lab or this website]

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This assay is applicable to any enzyme for which there is a fluorescently-labelled (MUB or MUC) substrate:

- β-D-cellubiosidase (CB)
- α-Glucosidase (AG)
- β-Glucosidase (BG)
- L-Leucine-7-amidomethylcoumarin (LAP)
- N-acetyl-β-glucoasminidase (NAG)
- Phosphatase (PHOS)
- β-Xylosidase (XYL)

Equipment

- 15 mL centrifuge tubes
- Pipetting reservoirs (one for each standard concentration (6) x two standards = 12 + one for each substrate = 18 for all 6 substrates)
- Centrifuge equipped with holders for deep-well plates
- Deep-well plates with lids (8 plates for 12 samples—4 each for samples & standards at 4 temps.)
- Waring Blender with small stainless steel blending cup
- Small glass dishes
- Magnetic stir bars and stir plate
- 8-channel repeat pipettor (volume distribution at least 200uL-800uL)

Reagents & Solutions

- 4-MUB-B-D- xylopyranoside: Sigma M7008 (100mg)
- 4-MUB-β-D-glucopyranoside: Sigma M3633 (250mg)
- 4-MUB-α-D-glucopyranoside: Sigma M9766 (100mg)
- 4-MUB-N-acetyl- β -D-glucosaminide: Sigma M2133 (100mg)
- 4-MUB- β -D-cellobioside: Sigma M6018 (100mg)
- 4-MUB phosphate: Sigma A8625 (25g)
- L-leucine-7-amido-4-methylcoumarin hydrochloride: Sigma L2145 (25 mg)
- 7-amino-4-methylcoumarin (MUC): Sigma A9891 (250mg)
- 4-methylumbelliferone (MUB): Sigma M1381
- 50 mM Buffer
  * Buffer pH should match the soil pH as closely as possible
    - for acidic soils → sodium acetate buffer
    - for basic soils → Tris buffer
  * http://www.bioinformatics.org/JaMBW/5/4/index.html is a good buffer calculator

Fluorescent Substrate Preparation (200 µM)

Dissolve the fluorescent substrates in deionized water according to the following list:

- β-Xylosidase4-MUB-β-D- xylopyranoside: 6.17 mg/100 mL DI H_2O
- BG: 4-MUB-β-D-glucopyranoside: 6.77 mg/100 mL DI H_2O
- AG: 4-MUB-α-D-glucopyranoside: 6.77 mg/100 mL DI H_2O
- NAG: 4-MUB-N-acetyl- β -D-glucosaminide: 7.59 mg/100 mL DI H_2O
- CB: 4-MUB- β -D-cellobioside: 10 mg/100 mL DI H_2O
- Phosphatase: 4-MUB phosphate: 5.12 mg/100 mL DI H_2O
- LAP: L-leucine-7-amido-4-methylcoumarin hydrochloride: 6.5 mg/100 mL DI H_2O

Stir and use moderate heat to speed dissolution of substrates.

Aliquots: Put 11 mL aliquots into 15 mL centrifuge tubes.

Storage: Can be stored frozen (-20°C for up to two months). Thaw in warm water. Do not thaw and refreeze

Stock Standard Solution Preparation (1 mM)

Dissolve 17.6 mg of MUB in 100 mL DI H_2O (use a volumetric flask).
Dissolve 17.5 mg of MUC in 100 mL DI H_2O (use a volumetric flask).

Aliquots: Put 5 mL aliquots into 15 mL centrifuge tubes.

Storage: Can be stored frozen (-20°C for up to two months). Do not thaw and refreeze.

Standard Curve

- Dilute standard stock solution (1 mM=1000 µM) to create a standard curve. Start with a 1:10 dilution of the stock solution to create a 100 µM solution. Make all further dilutions from 100 µM. Aim for 30 mL of each standard for a typical assay run with 12 samples.
  100 µM = 3 mL 1000 µM stock into 27 mL DI H_2O (1:10 dilution)
  50 µM = 15 mL 100 µM into 15 mL DI H_2O (1:2 dilution)
  25 µM = 15 mL 50 µM into 15 mL DI H_2O (1:2 dilution)
  10 µM = 12 mL 25 µM into 18 mL DI H_2O (1:10 dilution)
  5 µM = 15 mL 10 µM into 15 mL DI H_2O (1:2 dilution)
  2.5 µM = 15 mL 5 µM into 15 mL DI H_2O (1:2 dilution)
- To make different amounts of standard, use the following formula:

  (High Concetration)(x) = (Low Concentration)(Desired Volume)

  x = volume of the higher concentration to add to water
  add to volume of water = (desired volume – volume of higher concentration)

  e.g. To make 80 mL of 25 µM from 50 µM:

  (50 µM)(x) = (25 µM)(80 mL)

  x = 40 mL of 50 µM in 40 mL H_2O

- Because of background autofluorescence, a standard curve must be prepared for each sample. See plate layout for clarification.

Buffer Preparation

- The buffer you select will depend on the pH of the soil. Below are the instructions for two common buffers: sodium acetate (pKa = 4.76) and Tris (pKa = 8.06).
  - Note that phosphate buffer has a pKa = 7.2 which could work for more neutral/slightly basic soils. However, high phosphate concentrations may interfere with enzyme activity so use with caution.

50 mM Sodium Acetate Buffer

1. Dissolve 30.8 g sodium acetate in 2L DI H_2O
2. Adjust the pH to match soil pH using glacial acetic acid or concentrated sodium hydroxide (~10M NaOH).
3. Bring the volume up to 4L with DI H_2O.

Storage: Buffer should be made fresh, but can be refrigerated (4°C) for up to a week.

50 mM Tris Buffer

* notes on this are in my notebook – will add later!

Logistics

- Standards and substrates should be prepared well ahead of time.
- On the morning of the assay:
  - Thaw substrates and required standards (MUC only needs to be thawed if you are doing the LAP assay).
  - Prepare buffer.
  - Prepare standard curve (see below for dilutions)
- This method is best done with two people (one preparing soil slurries and the other preparing plates). Two people can easily prepare assays for up to **12 soil samples** in a morning (at most 2 hrs).
- Expert practitioners can start two sequential sets of assays in a morning. If you start assays in the afternoon, you will likely have to come in late to read the 15°C plates. Be sure to note incubation times for temperature of interest:

Temperature	Time
4°C	~ 23 hours
15°C	6 hours
25°C	3 hours
35°C	1.5 hours

- Preparing samples to be read on the microplate reader takes one person approximately 10 min. per plate. Total analysis time is typically 1.5 hours over the course of a day for a full set of samples.

Tips & Tricks

- You may need to alter the plate layout for your experiment. However, we've provided the plate layouts we use for doing all the enzymes at once.
- Because of differences in background autofluorescence, you must **prepare a standard curve for each sample** separately, hence the large standard plates.

- When starting, add the standard solutions prior to slurry addition. That way when you are finished adding soil samples the standard plates are ready to be incubated right away and can be read on the microplate before the assay plate. If you don't do this then there will a time crunch because at 25°C and 4°C, you will need to measure two standard plates and an assay plate at almost the same time and it takes ~10 minutes per plate, so you want them spread out.
- Do NOT add the substrates until after all the soil slurries have been added to the plate, otherwise you'll have uneven incubation time.
- The blank graphical representation of the plate may also be useful.

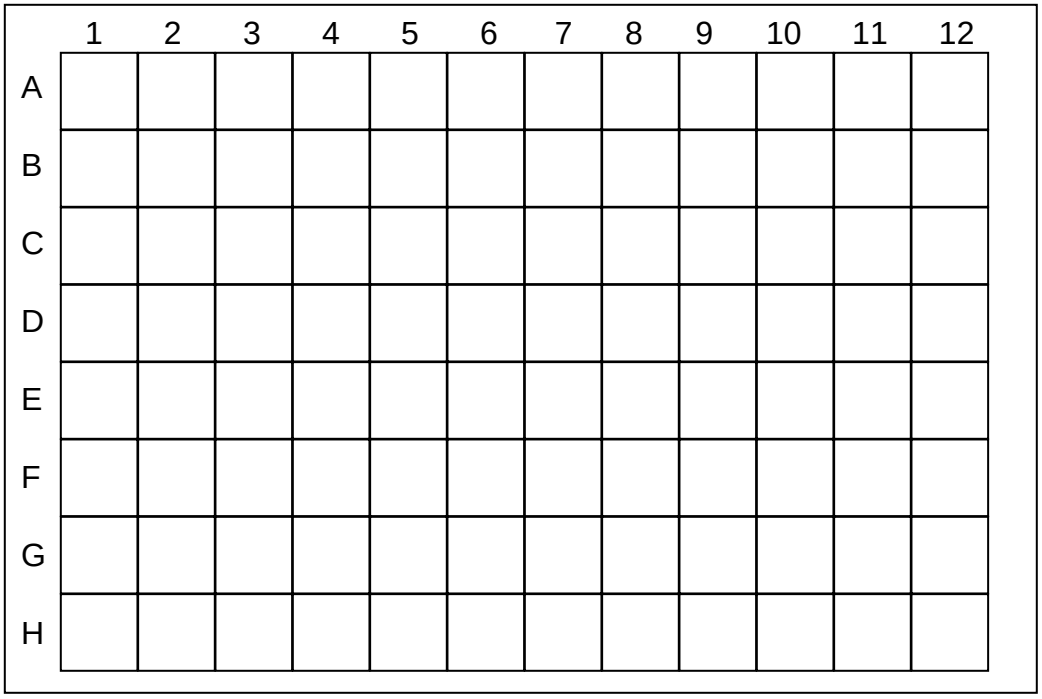

Plate Layouts:

Enzyme Activity Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1+ BG	S2+ BG	S3 + BG	S4+ BG	S5+ BG	S6+ BG	S7+ BG	S8+ BG	S9+ BG	S10+ BG	S11+ BG	S12+ BG
B	S1+CB	S2+CB	S3+CB	S4+CB	S5+CB	S6+CB	S7+CB	S8+CB	S9+CB	S10+CB	S11+CB	S12+CB
C	S1+ NAG	S2+ NAG	S3+NAG	S4+ NAG	S5+ NAG	S6+ NAG	S7+ NAG	S8+ NAG	S9+ NAG	S10+ NAG	S11+ NAG	S12+ NAG
D	S1+PHOS	S2+PHOS	S3+PHOS	S4+PHOS	S5+PHOS	S6+PHOS	S7+PHOS	S8+PHOS	S9+PHOS	S10+PHOS	S11+PHOS	S12+PHOS
E	S1+ XYL	S2+XYL	S3+ XYL	S4+XYL	S5+XYL	S6+XYL	S7+XYL	S8+XYL	S9+XYL	S10+XYL	S11+XYL	S12+XYL
F												
G												
H	S1 +LAP	S2 +LAP	S3 +LAP	S4+LAP	S5+LAP	S6+LAP	S7+LAP	S8+LAP	S9+LAP	S10+LAP	S11+LAP	S12+LAP

4 and 25C Standards **Same for MUB and MUC**

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1+ std0	S2+ std0	S3+ std0	S4+ std0	S5+ std0	S6+ std0	S7+ std0	S8+ std0	S9+ std0	S10+ std0	S11+ std0	S12+ std0
B	S1+std2.5	S2+std2.5	S3+std2.5	S4+std2.5	S5+std2.5	S6+std2.5	S7+std2.5	S8+std2.5	S9+std2.5	S10+std2.5	S11+std2.5	S12+std2.5
C	S1+ std5	S2+ std5	S3+ std5	S4+ std5	S5+ std5	S6+ std5	S7+ std5	S8+ std5	S9+ std5	S10+ std5	S11+ std5	S12+ std5
D	S1+std10	S2+std10	S3+std10	S4+std10	S5+std10	S6+std10	S7+std10	S8+std10	S9+std10	S10+std10	S11+std10	S12+std10
E	S1+ std25	S2+ std25	S3+ std25	S4+ std25	S5+ std25	S6+ std25	S7+ std25	S8+ std25	S9+ std25	S10+ std25	S11+ std25	S12+ std25
F	S1+ std50	S2+ std50	S3+ std50	S4+ std50	S5+ std50	S6+ std50	S7+ std50	S8+ std50	S9+ std50	S10+ std50	S11+ std50	S12+ std50
G	S1+ std100	S2+ std100	31+ std100	S4+ std100	S5+ std100	S6+ std100	S7+ std100	S8+ std100	S9+ std100	S10+ std100	S11+ std100	S12+ std100
H												

- The spaces in the enzyme activity sample plate at rows F and G are there in case other MUB based enzymes need to be measured at some point.
- S1…S12 = samples 1-12
- std0…std100 = standard concentrations

Procedure

I. Assay Set-Up

1. Prepare standard plates by pipetting 200 µL of appropriate standards into correct wells of MUB or MUC standard plates (oriented in *rows*).
   * When you add soil in Step 5, record the time and read these plates after the same amount of elapsed time as the enzyme assay plates at the corresponding temperature.
   • Prepare two plates for each standard (MUB & MUC) for a total of four plates. One set is incubated at 4°C and the other at 25°C.
   • The 25°C standard curve is used for all but the 4°C assay (15°C, 25°C & 35°C).
2. Prepare soil slurries by weighing 2.75 grams of field moist soil into blender.
   • The soil amount can be altered, just be sure to adjust the buffer amount proportionally.
   • Be sure to measure the gravimetric water content so that dry weight (d.w.) can be calculated—enzyme activity is typically presented on a g^{-1} soil d.w. basis.
3. Add 91 mL of 50 mM buffer.
4. Blend contents on high for 1 min.
5 Pour contents into a glass bowl, add stir bar and place on stir plate. Mix on low.
   • Bowl must be wide enough to accommodate the 8-channel pipette.
   • Stirring gently helps keep soil suspended and minimize variation due to different amounts of soil present in each well.
6. Pipette 800 µL of soil slurry into wells for enzyme activity measurement and standard curves (see plate layouts). Each sample should be in a separate *column*.
7. Rinse blender with DI H_2O or buffer between samples. Repeat Steps 2-7 for each sample.
8. Pipette 200 µL of appropriate 200 µM substrate into correct assays wells (oriented in *rows*).
   * Record the time of substrate addition and try to start all plates/substrates as quickly as possible.

Note: Steps 1 & 8 are mostly easily performed with only six (6) pipette tips loaded on the repeat pipette (two additions across the plate for a total of 12).

II. Incubation & Analysis

1. Seal the plates with plate mats and mix by hand (invert about 10 times).
2. Place plates in appropriate incubators for required incubation period.

Temperature	Time
4°C	~ 23 hours
15°C	6 hours
25°C	3 hours
35°C	1.5 hours

3. When incubation is complete, centrifuge plates for 3 min. at 1500 rpm (~350 x*g*).
4. Transfer 250 µL from each well into corresponding well in a flat-bottomed black 96-well plate.
5. Add 5 µL of 0.5N NaOH to each sample.
   * Want final pH in wells to be ~10.
   • Adjust normality as necessary for pH of soil.

   Note: this step may be omitted without significant loss of sensitivity in high pH soils.

6. Following manufacturer's instructions for your plate reader, measure fluorescence using the following parameters:
   Excitation Wavelength = 365
   Emission Wavelength = 450

   Additional instructions for the Tecan Infinite M200 microplate reader:
   1. Select 10 reads but no multiple reads per well.
   2. Open Excel and make sure the correct destination file is open.
      - The Tecan will put the data in a new worksheet (tab) in whatever spreadsheet is open. Label each tab or put descriptive information in each worksheet as it is generated. Information needed, sample #s, MUB or MUC sample, MUB or MUC standard, gain of the microplate reader. If you are using numbers that don't mean anything, then do a summary sheet on each workbook that explains the numbers.

      e.g. Sample 1 is plot 25 0-5cm, Sample 2 is plot 25 5-15cm, etc…
   2. Run the standard plate at optimal gain. The highest measured value should be for the 100 µM standard. Note the optimal gain determined by the machine. If you are doing the 35°C temp then you won't have the standard information yet, so be careful, you may need to drop the gain more than 5 units. I would suggest dropping it 5 units, run the sample and then drop it again 5 units and run the sample again. That way when you run the 25°C standards 1.5hr later you have another option if the 100 µM standard is still too high for the first 5unit gain drop (another trick of the trade).
   3. Set the gain manually to 5 units below the optimal gain from the standard plate for all other plates with the same soil samples.
      * The gain dramatically changes the measured values. However, it may be necessary to drop the gain even further for some samples if the value exceeds detection limits (indicated by a red OVER in the spreadsheet output). It is **CRITICAL** that the standard plates are read at the same gain as the samples else the sample values are **MEANINGLESS**.

Data Analysis & Calculations

- Plot standard curves (fluorescence on Y, standard conc. on X) in Excel for the MUB and MUC standards for each soil sample. Calculate the slope, intercept & r^2. Accept the standard curve if r^2 is >0.98.
  - We often do two standard curves for each set of standards to get better resolution for high activity and low activity enzymes, but this isn't strictly necessary as the standard curves are quite linear.
    - Low Activity: plot 0-25 µM standards
    - High Activity: plot 10-100 µM standards
- Use y=mx+b to solve for unknown concentrations (x), where:
  y = sample fluorescence
  x = enzyme concentration
  m = slope from standard curve
  b = intercept from standard curve

  sample enzyme concentration = (sample fluorescence – std. curve intercept) ÷ std. curve slope

- Calculating the enzyme activity:

  Enzyme Concentration/Length of Incubation (h)/soil dry weight (g) = µmol activity/g soil d.w./h

Notes

Safety

- This is a relatively benign analytical technique. Wear gloves if you are sensitive and consider safety glasses.