HYDROLASE ASSAYS WITH COUMARIN SUBSTRATES
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ENYZMES	**SUBSTRATES**
Aminopeptidases	L-Alanine-7-amido-4-methylcoumarin
	L-Arginine-7-amido-4-methylcoumarin
	Asparagine-7-amido-4-methylcoumarin
	Aspartic acid-7-amido-4-methylcoumarin
	L-glutamic acid γ-7-amido-4 methylcoumarin
	Glycine-7-amido-4-methylcoumarin
	L-Leucine 7-amido-4-methylcoumarin
	L-proline-7-amido-4-methylcoumarin
	L-serine-7-amido-4-methylcoumarin
	L-tyrosine-7-amido-4-methylcoumarin
	L-pyroglutamic acid-7-amido-4-methylcoumarin
Esterases	4-MUB-p-guanidinobenzoate
	4-MUB - phosphate
	4-MUB - sulfate
	4-MUB - acetate
Glycosidases	4-MUB-α-L-Arabinopyranoside
	4-MUB-β-D-cellobioside
	4-MUB-α-D-galactoside
	4-MUB-β-D-galactoside
	4-MUB-α-D-glucoside
	4-MUB-β-D-glucoside
	4-MUB-β-D-glucuronide
	4-MUB-N-acetyl-β-glucosaminide
	4-MUB-β-D-mannopyranoside
	4-MUB-β-D-xyloside

NOTE: There are probably two or three dozen other coumarin linked substrates commercially available. The list here includes those that are available at modest prices. There are many MUB linked fatty acid substrates available at modest prices but most are not very soluble so only the acetate substrate is listed. Designing your own profile is a function of number of samples to be processed, resources available and the nature of your question.

OTHER REAGENTS:
Distilled or deionized water (e.g. Nano-pure, Milli-Q), preferably sterile.
Standards: Prepare 100 µM solutions of 4-methylumbelliferone and 7-amino-4-methylcoumarin in sterile water. Dilute to 10 µM.

For surface water samples: Bicarbonate buffer: Make a 100 mM stock solution by dissolving 8.4 g $NaHCO_3$ in 1.00 L of milli-Q water. Stock solution pH is 8.2. Dilute 50 mL of stock solution to 1.00 L with milli-Q water to make 5 mM solution.

PROCEDURE:

1. Prepare substrate stock solutions in sterile water.

Substrates:	Amount needed to make 100 ml of **200 μM** stock solutions in sterile water (*unless indicated differently)
L-Alanine-7-amido-4-methylcoumarin	7.21mg
4-MUB-α-L-Arabinopyranoside	6.17 mg
L-Arginine-7-amido-4-methylcoumarin	6.63 mg
Asparagine-7-amido-4-methylcoumarin	8.07 mg
Aspartic acid-7-amido-4-methylcoumarin	5.81 mg
4-MUB-β-D-cellobioside	10.00 mg
4-MUB-α-D-galactoside	6.77 mg
4-MUB-β-D-galactoside	6.77 mg
4-MUB-α-D-glucoside	6.77 mg
4-MUB-β-D-glucoside	6.77 mg
4-MUB-β-D-glucuronide	7.05 mg
4-MUB-β-D-mannopyranoside	6.77 mg
L-glutamic acid γ-7-amido-4 methylcoumarin **(100uM)**	3.05 mg*
Glycine-7-amido-4-methylcoumarin	6.26 mg
4-MUB-p-guanidinobenzoate	7.48 mg
L-Leucine 7-amido-4-methylcoumarin	6.50 mg
4-MUB-N-acetyl-β-glucosaminide	7.59 mg
4-MUB-phosphate	5.12 mg
L-proline-7-amido-4-methylcoumarin	7.06 mg
L-pyroglutamic acid-7-amido-4-methylcoumarin	5.73 mg
L-serine-7-amido-4-methylcoumarin	5.97 mg
4-MUB-sulfate	5.89 mg
L-tyrosine-7-amido-4-methylcoumarin **(100uM)**	3.38 mg*
4-MUB-β-D-xyloside	6.17 mg

Note: Once prepared in sterile water, substrate solutions appear to be stable for weeks if not contaminated. Some substrates, notably esterase substrates like MUB acetate, are highly sensitive to contamination. Punctilious care is needed to prevent spurious results. Use gloves, sterile tips, etc. It can be difficult to measure fatty acid esterase activity in some systems because it is so high.

The sections that follow provide guidelines for doing assays with various categories of environmental samples.

SURFACE WATER ASSAYS

NOTE: The following procedure will work for water of moderate to high alkalinity, pH> 7.5. Modifications for assaying water samples at pH < 7.5 are also presented.

1. Set up positive and negative controls: Dispense 50 µL of substrate and standard solutions and 200 µL of buffer solution into microplate wells, following the template you designed, to create reference standards, quenched standards, substrate controls, and sample controls. A description of these controls is given below.

Note: Each substrate and all controls should be replicated at least three times on each microplate. Plates can be prepared ahead of time and stored by covering and freezing them. Our results show that frozen plates produce responses very similar to those of freshly prepared plates. Your plate layout should minimally include:

- 3 replicate wells for each quenched standard. Quenched standard = 50 µL standard + 200 µL sample. Standard = 10 µM 4-methylumbelliferone or 7-amino-4 methyl coumarin.

- 3 replicate wells for each reference standard. Reference standard = 50 µL standard + 200 µL 5 mM bicarbonate buffer.

- 3 replicate wells for each substrate control. Substrate control = 50 µL substrate + 200 µL buffer.

- 3 replicate wells for each sample control. Sample control = 50 µL buffer + 200 µL sample.

- 3 replicate wells for each assay. Assay = 50 µL substrate + 200 µL sample

2. Pipette 200 µL of water sample to all sample control and assay wells. **NOTE**: Well A1 is generally used as a reference by the plate reader. Put 250 µL of 5 mM bicarbonate buffer in well A1.

3. Add 50 µL substrate solution to all assay wells. Record the time.

NOTE: If you are doing lots of assays or lots of samples concurrently. Best strategy is to set up all the plates first. Do everything except the addition of substrate to assay wells. When all the plates are set up, then begin adding substrate solutions to assays wells.

4. Warm up microplate fluorometer for 0.5 hr. Set excitation wavelength at 365 nm and emission wavelength at 450 nm. Read the microplate as soon as possible after sample addition. Record the time. Read the plate again at intervals of 0.1 - 1.0 h depending on activity.

Note: Some assays such as fatty acid esterase and phosphatase may be rapid; results

may be obtained within a few minutes. Activity against other substrates will be weak; the plates will need to incubate for several hours to get detectable activity.

Note: If the pH of your water sample is >7.5 you can read fluorescence without modifying the wells. The nanomolar emission coefficient of 4-methyumbelliferone and 7-amino-4-methyl courmarin is low at pH values < 7 and increases rapidly as you move to pH 9. The standards included on each plate permit calculation of the emission coefficient for your samples. If the pH of your water sample is < 7.5, you can either raise it before doing your assays, by adding bicarbonate, or run your assays at ambient pH. If you do the latter, it will be necessary to add a 10 µL aliquot of 0.5 N NaOH to each well (assays, standards, controls) at the termination of incubation in order to obtain fluorescence readings. In this situation, each well can only be read one time so preliminary trials may be required to determine appropriate incubation intervals.

4. After confirming that reaction rates were linear over the period of assay, calculate activity as nmol substrate converted per hour per mL of sample.

NOTE: If you have a choice of incubation intervals over which to calculate activity, it is generally best to calculate the "initial" rate of activity using the shortest interval you have that shows a measurable response. If you assayed your samples at low pH, each well can only be read once (after addition of NaOH) so you cannot determine whether reaction rate was linear through time. Generally this is not too important, but if you must know, you need to set up a series of wells that can be destructively assayed at different time intervals.

Activity (nmol h^{-1} mL^{-1}) = (mean final Flourescence - mean initial Fluorescence) / (emission coefficient)(quench coefficient)(0.2 mL)(incubation interval, hr)

Emission coefficient (fluor / nmole) = (mean fluorescence of reference standard) / 0.5 nmole

Quench coefficient = (mean fluorescence of standard + sample) / (mean fluroescence of standard + buffer)

NOTE: If you do not care about quantifying quench effect, just calculate an nanomolar extinction coefficient from the wells that contain water sample + standard, them simplify the equation above to:

Activity (nmol h^{-1} mL^{-1}) = (mean final Flourescence - mean initial Fluorescence) / (emission coefficient for quenched standard)(0.2 mL)(incubation interval, hr)

NOTE: If the negative controls (buffer + substrate solution) show increases in fluorescence over the incubation interval, it may be necessary to subtract the change in fluorescence by the negative controls from the net increase in fluorescence that you attribute to enzyme activity. In our experience, the negative controls are stable. There can be problems with the esterase substrates, but this generally stems from

contamination rather than substrate instability.

NOTE: If you assayed your samples at low pH, activity is calculated somewhat differently because you have only "final" readings not intial and final readings.

Activity (nmol h^{-1} mL^{-1}) = (mean Flourescence of assay wells - (mean substrate control fluor + mean sample control fluor))/(emission coefficient)(quench coefficient)(0.2 mL)(incubation interval, hr)

SOIL ASSAYS

The procedure for soil assays is similar to the one presented above for surface water samples with a pH lower than 7.5. The added complication is the particles which increase quenching and increase well to well variation.

1. Sample preparation: Place the equivalent of 1.0 g dry mass of soil into a 125 mL screw-cap Nalgene bottle. Add about 60 mL of 50 mM pH 5 acetate buffer to the bottle. Homogenize the soil sample using the polytron. Add additional acetate buffer to fill the bottle.

NOTE: A soil suspension of around 1 g/100 mL quenches fluorescence by 20-40%.

2. Shake the sample suspension vigorously and pour it into a round wide mouth dish or beaker. Add a stir bar and stir hard to maintain a uniform suspension. Use an 8-channel pipetter to withdraw 200 µL aliquots of the sample suspension and dispense them into a black microplate.

NOTE: Strong mixing and good pipetting technique will minimize well to well variation. Use wide mouth pipette tips or snip off the ends of standard tips. A larger diameter opening will improve uniformity of dispensing.

3. Assay wells: add 50 µL of substrate solution to an eight well column containing 200 µL of sample suspension. Sample control wells get 50 µL of acetate buffer + 200 µL of sample suspension. Substrate control wells get 50 µL substrate solution plus 200 µL of acetate buffer. Quench standard wells get 50 µL of standard (4-methylumbelliferone or 7-amino-4- methyl coumarin) + 200 µL sample suspension. Reference standard wells get 50 µL of standard (4-methylumbelliferone or 7-amino-4- methyl coumarin) + 200 µL acetate buffer.

NOTE: In this design, there are eight replicate wells for assays, standards, controls and blanks (compared to only three in the surface water procedure). Even with good technique, the well to well variation will be 20% or more because of differences in the amount of material hitting each well and because you are measuring changes in fluorescence against a high quench background. When scaling assays down from test tube to microplate, you sacrifice precision for economy. Depending on the nature of your samples and the magnitude of difference you consider significant, you may want to increase the number of replicate wells to 16. In our "standard" design, we use one

column of wells for each standard and control and two columns of wells for the assays.

NOTE: As an organizational hint, it is generally simpler to do only one type of assay per plate, that is designate one plate for phosphatase and another for glucosidase, etc., then run several different samples but only one assay on each plate. As noted in the protocol for surface waters, best strategy is to set up all the plates first by dispensing everything except substrate solution to assay wells. Then start the incubations by adding substrate to assay wells.

4. Incubate plates at 20 C. Depending on your question, you may chose to incubate at ambient temperature or at an elevated temperature.

5. To measure fluorescence it is necessary to raise the pH above at least 7.5. Raise pH by add a 10 µL aliquot of 0.5 N NaOH to each well (assays, standards, negative controls, blanks) at the termination of incubation. In this situation, each well can only be read one time so preliminary trials may be required to determine appropriate incubation intervals or else a series of wells will have to set up for series of destructive fluorescence readings at appropriate time intervals.

Note: Some assays such as N-acetylglucosaminidase and phosphatase may be rapid; results may be obtained within a few minutes. Activity against other substrates will be weak; the plates will need to incubate for several hours to get detectable activity.

NOTE: In this protocol, the assays are incubated at something close to the ambient pH of most soils. Buffer composition can be changed for a better match. Glycosidases have pH optima in the 4-6 range, so the acetate buffer "optimizes" those assays. Peptidases generally have pH optima around 8, so these activities are discriminated against in this procedure. Phosphatases can have acid or alkaline pH optima, so profiles generally show high activity across a broad range of pH. If desired, a separate buffer system can be used for each type of enzyme to facilitate detection. A tris-hydroxymethyl aminomethane buffer will work at pH 8. If you run assays at pH 8 or above, it will be possible to read fluorescence directly and repeatedly without the addition of NaOH.

6. Calculate activity as nmol substrate converted per hour per mL of sample.

Activity (nmol h^{-1} g^{-1}) = (mean Flourescence of assay wells - mean Fluorescence of negative control wells - mean Fluorescence of blank wells) (125 mL) / (emission coefficient)(quench coefficient)(0.2 mL)(incubation interval, hr)(g soil used to make suspension)

Emission coefficient (fluor / nmole) = (mean fluorescence of reference standard) / 0.5 nmole

Quench coefficient = (mean fluorescence of quenched standard) / mean fluroescence of reference standard)

125 is the total volume of sample suspension

0.2 is the volume of sample suspension in each well

LITTER OR POM ASSAYS

Follow the protocol for soils with the following considerations:

Litter or POM needs to be thoroughly mixed prior to subsampling for assay. Depending on the material, use some combination of milling or blending or chopping to get pieces down to a size where the polytron. A standard blender will work for material that is relatively dry. Scissors work too. The material needs to broken into fragments of 1 cm or less for representative subsampling. Also the polytron will not be able to homogenize particles larger than 1 cm. It is critical to assay replicability that the particles are homogenized to small size.

Litter or POM will have much higher activities than surface waters or soil. Use only 0.5 g of material rather than 1.0 g listed in the soil assay protocol. Incubations times may be much shorter. Sometimes only a few minutes are needed for phosphatase or β-glucosidase assays.