Assays with p-Nitrophenyl linked Substrates

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1. PREPARE SUBSTRATE SOLUTIONS.

For carbohydrase assays, dissolve substrates in 50 mM, pH 5, acetate buffer.

For peptidase assays, dissolve substrates in 50 mM, pH 8, Tris buffer.

For acid phosphatase, use acetate buffer, for alkaline phosphatase use a pH 9 carbonate buffer.

Default substrate concentration is 5.0 mM.
NOTE: Some substrates are not soluble enough to make 5 mM solutions. Others are too expensive. The goal is to conduct assays under conditions of substrate saturation. In general, 5 mM solutions are sufficient. However, when analyzing unfamiliar samples or under conditions of insufficient particle homogenization or insufficient mixing during incubation it may be necessary to use higher substrate concentrations to assure zero order kinetics.

Assay	**Substrate**
phosphatase	pNP-phosphate
β-glucosidase	pNP-β-glucopyranoside
cellobiohydrolase (CBH)	pNP-cellobioside *
chitinase	pNP-β-N,N'-diacetylchitobiose **
α-glucosidase	pNP-α-glucoside
β-xylosidase	pNP-β-xylopyranoside
β-N-acetylglucosaminidase (NAG)	pNP-β-N-acetylglucosaminide ***
leucine aminopeptidase	leucine p-nitroanilide ***
glycine aminopeptidase	glycine p-nitroanilide
"trypsin" peptidase	pNP-p'-guanidino-benzoate

* expensive, try 2 mM
** very expensive, use 2 mM
*** not very soluble, use 2 mM

Substrate solutions can be made up in 100 or 200 mL batches and stored in the refrigerator for up to a few weeks if uncontaminated.

Check the pH of the substrate solutions. Some will require pH adjustment after mixing. In particular, pNP-phosphate will depress pH by 1-2 units.

2. MIX 2.0 ml OF SAMPLE WITH 2.0 ml OF SUBSTRATE SOLUTION IN A 5 ml POLYPROPYLENE TUBE. CAP TUBES, PLACE THEM IN ZIP LOCK BAGS, PUT BAGS IN THE PLATELET MIXER. INCUBATE FOR 1-6 hr AT 20 C.

NOTES: The sample is usually a suspension of fine particles or a homogenate of larger particles. When using suspensions, place the sample bottle on a stir plate, mix vigorously, withdraw 2 ml aliquots using a the P5000 pipetter. To prevent clogging of the tip, snip off the end to make an opening about 0.5 cm in diameter.

It is possible to scale down the assay mixture to 1.0 ml of sample and 1.0 of substrate solution if either sample or substrate quantities are limited.

Do 3-4 analytical replicates per sample.

Prepare sample controls by placing 2.0 mL of sample and 2.0 mL of acetate buffer in a tube and incubate it concurrently. Prepare substrate controls by mixing 2 mL of buffer with 2 mL of substrate solution. Prepare controls in duplicate.

If the sample is an extract or a bulk water sample, combine 1 mL of sample extract and 1 mL of substrate solution in a 15 mL test tube, vortex, incubate tubes at 20 C, then skip to step 4.

For most samples, incubation times of 1 h or less should be used for phosphatase. β-glucosidase activity is often high too. CBH and NAG assays usually need to incubated for several hours to generate significant product.

3. SPIN THE REACTION SUSPENSIONS FOR 2-3 min IN TABLE TOP CENTRIFUGE AND PIPETTE 2.0 ml OF SUPERNATANT INTO A 15 ml TEST TUBE.

4. ADD 0.2 ml OF 1.0 N NaOH TO EACH TEST TUBE TO TERMINATE THE REACTION AND DEVELOP THE COLOR.

5. ADD 8.0 ml OF DISTILLED WATER TO EACH TEST TUBE AND VORTEX.

6. READ ABSORBANCE OF EACH TUBE AT 410 nm. ZERO THE SPECTROPHOTOMETER WITH DISTILLED WATER.

NOTE: In general, if sample absorbances exceed 2.000 the assay should be repeated using a shorter incubation time.

7. ACTIVITY IS EXPRESSED IN µmol of substrate hydrolyzed per hour per g organic matter as follows:

OD = Sample Abs - [Substrate control Abs + Sample control Abs]

Activity ($\mu mol\ h^{-1}\ gOM^{-1}$) =

$$OD / [(1.590 / \mu mol)\ (incubation, hr)\ (g\ OM / mL\ sample\ homogenate)]$$

The micromolecular extinction coefficient for p-nitrophenol is 1.59 under the conditions of this assay. To calculate, run a standard curve by making dilutions of a 1.00 µmol / mL

solution of p-nitrophenol in buffer. Mix 1 ml of standard, 0.1 ml of 1.0 N NaOH, and 4 ml of distilled water. Read absorbances. Do a linear regression of OD vs. concentration. The slope of the line is the extinction coefficient. Absorbance is linear with concentration up to an OD of about 2.000.