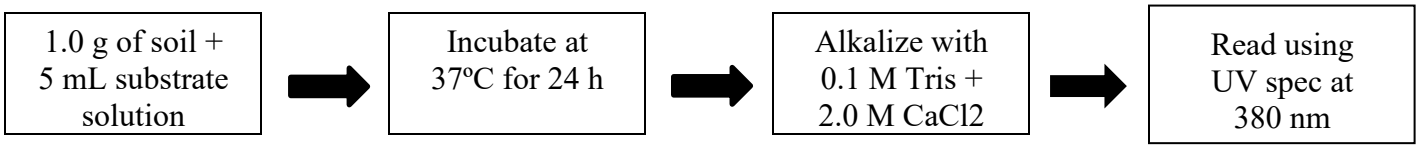
SOP: **Enzyme Assays: *p*NA**

Overview:

This standard operating procedure (SOP) describes a protocol for detecting several aminopeptidase rates of activity, using *para*-nitroanilide (*p*NA)-linked substrates. The method was originally reported by Tabatabai and Bremner (1969), and adapted by Allison and Vitousek (2005) and Daughtridge et al. (2021). Key instruments are a laboratory water bath, microcentrifuge, and ultra-violet spectrophotometer. Key safety considerations are the use of nitrile gloves and laboratory coat. Soils that are ground to pass a <2 mm sieve are typically used.



Safety:

All standard safety protocols and online safety training via UIUC Division of Research Safety (DRS) are required.
Personal protection (PPE) for this procedure include:
Body Protection: Laboratory coat
Hand Protection: Nitrile gloves
Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Substrate solution preparation

- Analytical balance capable of weighing to three decimal places
- 50 mL disposable polypropylene centrifuge tubes (Falcon tubes)
- Styrofoam centrifuge tube racks
- Metal stir bar and stir plate
- Laboratory glassware
- *p*NA (*para*-nitroaniline; FW=138.13)
- *p*NA-linked substrate (e.g. L-Leucine-p-nitroanilide for Leucine aminopeptidase activity)

Reagent preparation

- 0.1 M Tris, pH=12 (MW=121.1)
  - also called THAM, Trizma base
- pH probe
- NaOH (pellets or 0.5 M solution)

- 2.0 M $CaCl_2$ (MW=147.01)

Reaction

- 1 mL and 5 mL pipettor and tips
- Laboratory water bath capable of maintaining 37ºC for 24 hours
- Plastic wrap
- Metal/plastic tray

Alkalization

- Microcentrifuge and 1.5-2 mL microcentrifuge tubes
- 1 mL and 5 mL pipettor and tips

Spectrophotometry

- 200 µL-1 mL pipettor and tips
- Ultra-violet spectrophotometer
- 96-well microplates

Detailed Procedure:

I. Sample and substrate solution preparation

1. Measure 1 ± .01 g air-dried soil (for field-moist: 1 g oven-dry equivalent) into 50 mL Falcon tubes
    i. A maximum of 50 samples is recommended per set
2. Prepare 1 mM substrate solution in deionized water
    i. Some substrates may not dissolve in water, so suspension using a stir plate is recommended
3. Begin heating water bath to 37ºC

II. Reagent preparation

1. 0.1 M Tris, pH=12 (MW=121.1)
    i. Use a pH probe to raise the pH to 12 with 0.5 M NaOH
    ii. Store for up to 2 weeks at room temperature
    iii. Necessary to halt reaction
    iv. Evidence suggests that alkalization isn't needed for *p*NA color development (Alvarado et al. 1992). However, it may be useful for *p*NA extraction from the soil.
    v. Originally 0.5 M NaOH was used until evidence of its hydrolysis of substrates and thus potential overestimate of potential activities
    vi. Tris is also called THAM, Trizma base
2. 2.0 M $CaCl_2$ (MW=147.01)
    i. Flocculates soil
    ii. Store up to 6 months at room temperature
    iii. Although 0.5 M is the traditional concentration, 2.0 M is used to avoid DOM co-extraction with alkaline termination. See Margenot et al. (2018) for rationale.

III. Standard preparation

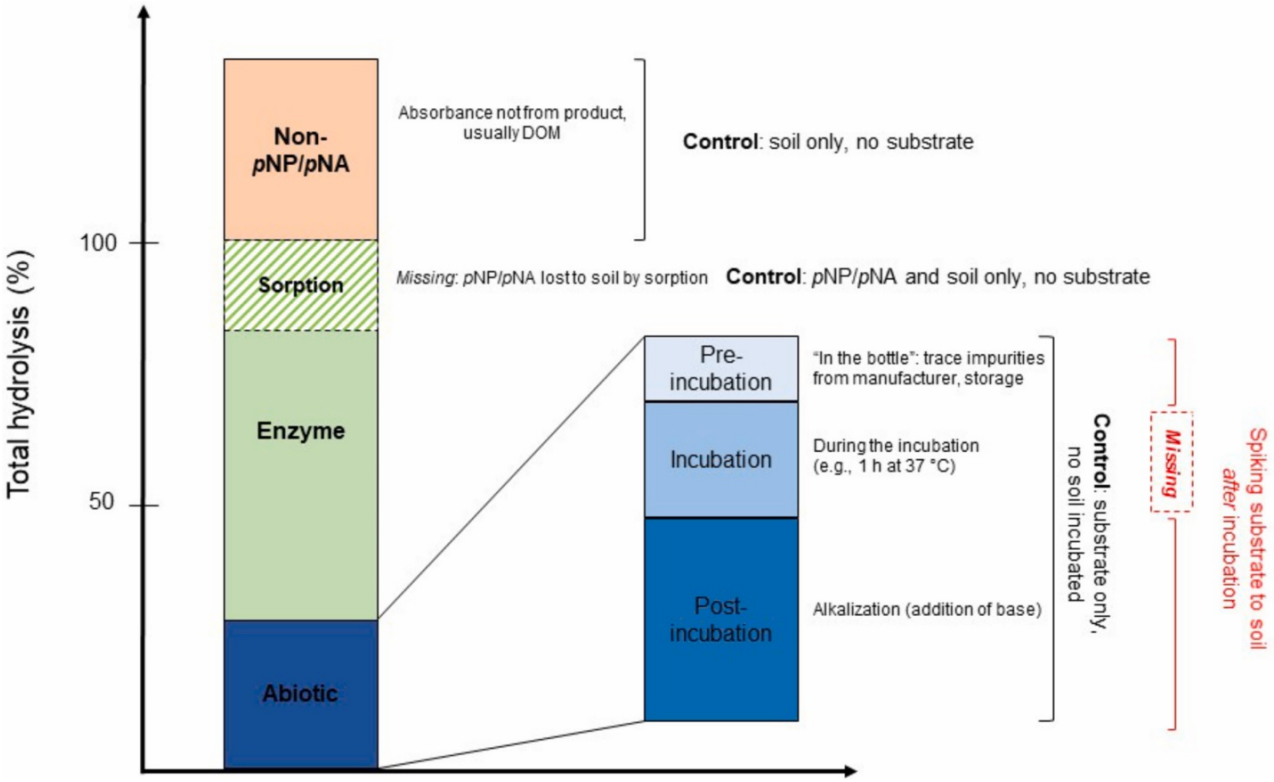
1. Dissolve 1 mM *p*NA in deionized water
    i. *p*NA will need 3-4 hours on a stir plate or shaker table to fully dissolve in water.
    ii. Stock solution is stable for at least 1 month when stored at 4ºC
2. Pipette 5 mL of *p*NA stock solution into a falcon tube, followed by 4 mL 0.1 M Tris and 1 mL 2.0 M $CaCl_2$
3. Using microcentrifuge tubes, dilute the alkalized standard solution as follows:

1x (1 mM)	1 mL stock	0 mL water
2x (0.5 mM)	0.25 mL stock	0.25 mL water
3x (0.333 mM)	0.25 mL stock	0.50 mL water
4x (0.25 mM)	0.25 mL stock	0.75 mL water
5x (0.20 mM)	0.25 mL stock	1.00 mL water
10x (0.10 mM)	0.1 mL stock	0.9 mL water
20x (0.05 mM)	0.25 mL 10x dilution	0.25 mL water

IV. Corrections

1. True Blank
    i. Necessary for detection of interference in UV spectrophotometer readings. This blank should also be used as the 0 mM *p*NA point on the standard curve. A blank with soil without substrate is used for correction.
2. Abiotic Hydrolysis
    i. Substrates are subjected to abiotic hydrolysis during storage, incubation, and alkalization. Blank with same *p*NA-substrate concentration as your assays with no soil should be added. These also start to precipitate, thus immediately measured after centrifugation. No dilution should be necessary if your substrate is in good quality. Please note that NaOH can induce higher abiotic hydrolysis of *p*NA-linked substrates.
3. Dissolved Organic Matter (DOM) Interference
    i. This is critical when you see dark brown-black color in your alkalized assay solutions even after centrifugation. A blank with soil without substrate is used for correction.
4. *p*NA Recovery
    i. This is critical for soils with high sorption capacity of *p*NA, and it is notable that many soils have a higher sorption capacity of *p*NA than *p*NP. Blank with *p*NA solution (not *p*NA-substrate) and soil should be used to correct for the recovery. The initial *p*NA solution you make for standards (water + *p*NA) may be used. The concentration

of *p*NA to be spiked should be close to the amount your soil hydrolyzes. Rate of recovery can be calculated by dividing the measured concentration corrected for dilution by the initial concentration of *p*NA.



V. Reaction

1. Uncap Falcon tubes. Caps are no longer needed.
2. Pipette 5 mL of 1 mM substrate solution into the tubes containing samples
3. Pipette 5 mL of 1 mM substrate solution in the tubes for abiotic hydrolysis corrections
4. Pipette 5 mL of deionized water into the tubes for DOM and true blank corrections
5. Pipette 5 mL of 1 mM *p*NA standard into the tubes for *p*NA recovery corrections
6. Swirl for 1 min, then incubate at 37ºC for 24 hours
    i. Place plastic wrap over the uncapped tubes and submerge them (bottom half of the tubes submerged) in the water bath pre-heated to 37ºC. Weigh down the tubes using a tray and 1-2 heavy objects (e.g. 0.5 L bottles of water)

VI. Alkalization

1. Once the samples and corrections are removed from the water bath, immediately pipette 4 mL 0.1 M Tris (pH=12) into each tube
2. Pipette 1 mL 2.0 M $CaCl_2$ into each tube; let sit ~5 min or until clear aliquot is evident

VII. Centrifugation and spectrophotometry

1. Pipette 1 mL of each sample/correction supernatant into microcentrifuge tubes
2. Cap and centrifuge for 1:45-2:00 min
3. Pipette 200 µL of the centrifuged supernatants into microplate wells, and read at 380 nm
    i. If sample or correction absorbance exceeds the range of the standard curve, dilution with water is needed. For 2x dilution, transfer 100 µL of supernatant to a new microplate well and add 100 µL water. Repeat for 4x dilution.

VIII. Clean up

1. Waste from Falcon tubes, microcentrifuge tubes, and microplates should be consolidated in a capped and labeled bottle.
2. Used but emptied Falcon tubes, microcentrifuge tubes, and microplates may be thrown away in regular trash bins.

IX. Calculations

Measurement of aminopeptidase activity is usually expressed in units of µmol *p*NA g^{-1} soil h^{-1}. To calculate, see corresponding Excel calculation template.

Example calculation:

Sample Absorbance = 1.384
Dilution = 1
Standard curve y = 0.2963x - 0.0351
*p*NA recovery = 0.8988
Abiotic hydrolysis blank absorbance = 0.348
DOM blank absorbance = 0.080

Convert absorbance to concentration
Sample conc. = 0.2963*1.384 - 0.0351 = 0.375 mM
Abiotic hydrolysis blank conc. = 0.2963*0.348 - 0.0351 = 0.068 mM
DOM blank conc. = 0.2963*0.080 - 0.0351 = -0.011 mM (Change to negative when this low)

Convert sample concentration to activity

$$Potential\ activity = \frac{\left(\frac{0.375mM * 1}{0.8988} - 0.068mM - 0.0mM\right) * 0.01L * \frac{1000\mu mol}{mmol}}{1g\ soil * 24hr}$$

$$= 0.1455\ \mu mol\ pNA\ g - 1\ soil\ hr - 1$$

References:

1. Tabatabai and Bremner 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry* 1:301. https://doi.org/10.1016/0038-0717(69)90012-1

2. Alvarado et al. 1992. Rapid p-nitroaniline test for assessing microbial quality of refrigerated meat. Journal of Food Science 57: 1330-1331. https://doi.org/10.1111/j.1365-2621.1992.tb06849.x

3. Allison and Vitousek 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biology and Biochemistry 37: 937-944. https://doi.org/10.1016/j.soilbio.2004.09.014

4. Margenot et al. 2018. Methodological recommendations for optimizing assays of enzyme activities in soil samples. *Soil Biology and Biochemistry* 125:350. https://doi.org/10.1016/j.soilbio.2017.11.006

5. Daughtridge et al. 2021. Sources of abiotic hydrolysis of chromogenic substrates in soil enzyme assays: Storage, termination, and incubation. *Soil Biology and Biochemistry* 158. https://doi.org/10.1016/j.soilbio.2021.108245

Suggested reading:

1. Allison et al. 2006. Elevated enzyme activities in soils under the invasive nitrogen-fixing tree Falcataria moluccana. Soil Biology and Biochemistry 38: 1537-1544. https://doi.org/10.1016/j.soilbio.2005.11.008

2. Burns et al. 2013. Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biology and Biochemistry* 58:216. https://doi.org/10.1016/j.soilbio.2012.11.009

3. Nannipieri et al. 2018. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. *Biology and Fertility of Soils* 54:11. https://doi.org/10.1007/s00374-017-1245-6

Citation:

SOP: Enzyme Assay: pNA. 2021. Soils Lab, University of Illinois Urbana-Champaign. Urbana, IL. Accessed at: https://margenot.cropsciences.illinois.edu/methods-sops/

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