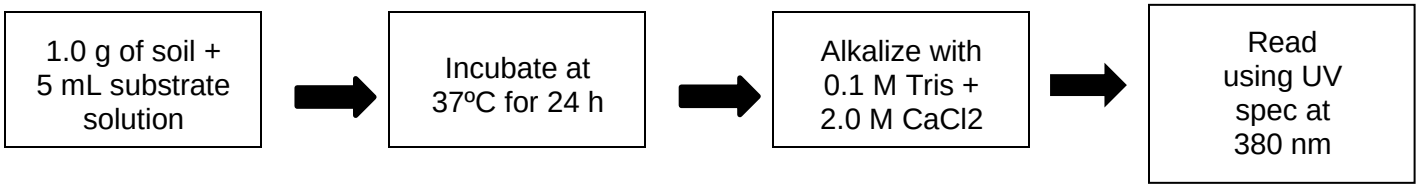
SOP: **Enzyme Assays: *p*NA**

Overview:

This standard operating procedure (SOP) describes a protocol for quantifying rates of activity of several aminopeptidases, 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-4-nitroanilide for Leucine aminopeptidase activity)

Reagent preparation

- 0.1 M Tris, pH 12.0 (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)

- pH meter

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. Substrate solution preparation

1. Prepare 1 mM *p*NA-substrate solution in deionized water (Table 1).
    i. Some substrates may not dissolve in water, so suspension using a stir plate is recommended.

Table 1. Soil enzyme activities that can be assayed with *p*NA-linked substrates, and commonly used assay conditions.

Abbr.	Enzyme	Substrate	Molar weight (g/mol)	[Subs] mM	Time (h)	CAS number
GAP	Glycine aminopeptidase	Glycine 4-nitroanilide	195.18	1	24	1205-88-5
LAP	Leucine aminopeptidase	L-Leucine 4-nitroanilide	251.30	1	24	4178-93-2
ALA	Alanine aminopeptidase	L-Alanine 4-nitroanilide hydrochloride	245.70	1	24	31796-55-1
ARG	Arginine aminopeptidase	L-Arginine 4-nitroanilide dihydrochloride	367.30	1	24	40127-11-5
MET	Methionine aminopeptidase	L-Methionine 4-nitroanilide	269.33	1	24	6042-04-2
PRO	Proline aminopeptidase	L-Proline *p*-nitroanilide trifluoroacetic acid salt	349.30	1	24	108321-19-3
LYS	Lysine aminopeptidase	L-Lysine *p*-nitroanilide dihydrobromide	428.12	1	24	40492-96-4
GLU	Glutamic acid aminopeptidase	L-Glutamic acid γ-(*p*-nitroanilide) hydrochloride	303.70	1	24	67953-08-6

ii. To determine the substrate mass (g) required for a given volume:

$$Substrate\ mass\ (g) = Volume\ needed\ (L) \times Substrate\ concentration\ \left(\frac{mol}{L}\right) \times Molar\ weight\ of\ substrate\ \left(\frac{g}{mol}\right)$$

II. Sample preparation

1. Measure 1.00 ± 0.01 g air-dried soil (for field-moist: 1 g oven-dry equivalent) into 50 mL centrifuge tubes
    i. A maximum of 50 samples is recommended per set

III. Reagent preparation

1. 0.1 M Tris, pH 12.0 (MW=121.1 g/mol)
    i. Use a pH meter to raise the pH to 12.0 using 0.5 M NaOH
    ii. Stable for up to 2 weeks at room temperature
    iii. Evidence suggests that alkalization is not needed for *p*NA color development (Alvarado et al. 1992). However, it may be helpful for *p*NA extraction from the soil.
    iv. Originally 0.5 M NaOH was used until evidence of its hydrolysis of substrates and thus potential overestimate of potential activities (Daughtridge et al., 2021).
2. 2.0 M $CaCl_2$ (MW=147.01)
    i. Flocculates soil
    ii. Stable up to 6 months at room temperature
    iii. Although 0.5 M is the traditional concentration**,** 2.0 M is used to avoid dissolved organic matter (DOM) co-extraction with alkaline termination. See Margenot et al. (2018) for rationale.

IV. Standard preparation

1. Make *p*NA-stock solution by mixing 0.0691 g of *para*-nitroaniline in 500mL of water to make 1 mM solution.
Note: para-nitroaniline takes 3-4 hours on a stir plate or shake to fully dissolve in water.

2. Transfer 5mL of *p*NA-stock solution to a new centrifuge tube, then add 1 mL $CaCl_2$ + 4mL Tris.

3. Dilute this solution (step 2) into the microcentrifuge tubes following the steps below.

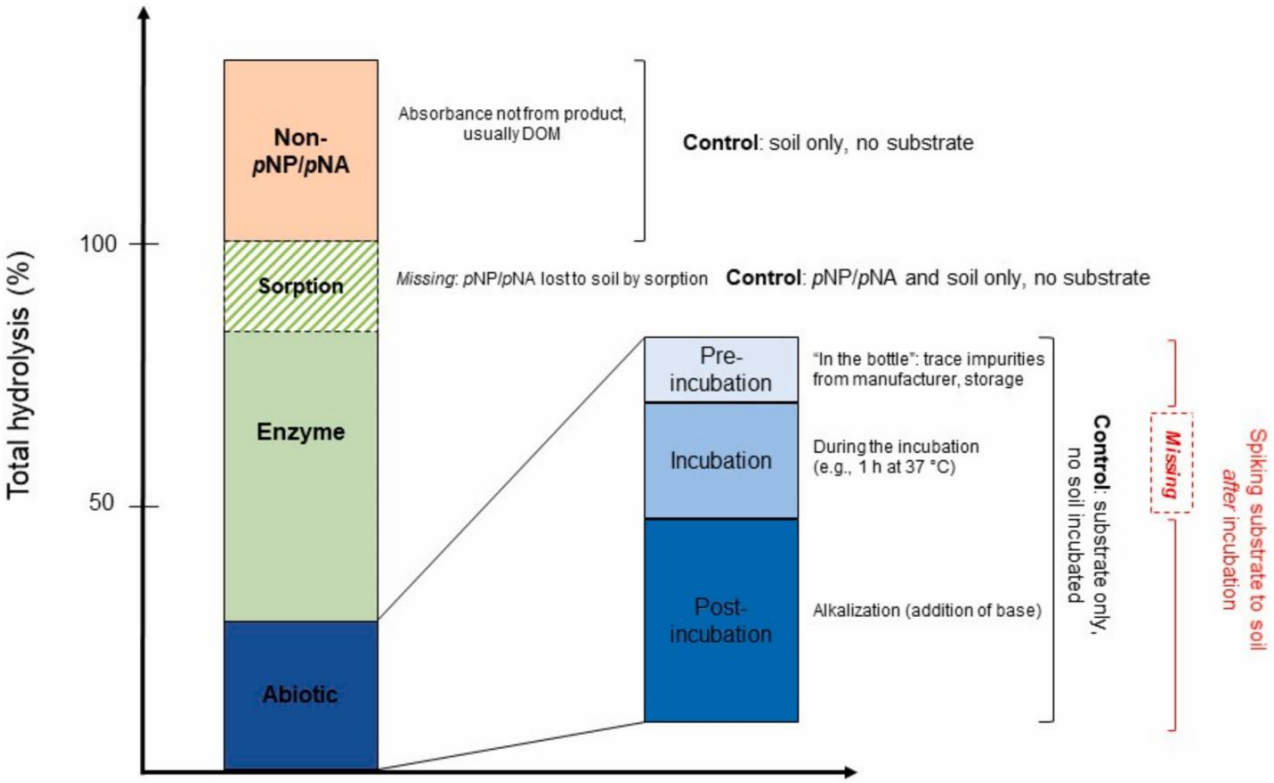
4. Hand shake tubes, then pipette 200 µL to well plate and read at 380 nm.

Dilution	Volume of standard (mL)	Volume of water (mL)	Concentration for curve (mM)
1x	1.00	0.00	0.500
2x	0.25	0.25	0.250
3x	0.25	0.50	0.167
4x	0.25	0.75	0.125
5x	0.25	1.00	0.100
10x	0.10	0.90	0.050
20x	0.25 (of the 10x)	0.25	0.025

V. Corrections

Prepare blanks that are going to be treated the same way as samples (sections VI to IX).

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.
    ii. In a 50 mL centrifuge tube, add 5 mL of water.
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.
    ii. In a 50 mL centrifuge tube, add 5 mL on *p*NA-substrate solution.
3. DOM Interference
    i. 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.
    ii. In a 50 mL centrifuge tube, add 1.00 g of soil and 5 mL of water.
4. *p*NA Recovery
    i. 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.
    ii. In a 50 mL centrifuge tube, add 1.00 g of soil and 5 mL of *p*NA-stock solution.



VI. Reaction

1. Uncap Falcon tubes. Caps are no longer needed.
2. Pipette 5 mL of water into an empty centrifuge tube (true blank).
3. Pipette 5 mL of *p*NA-substrate solution into samples and into the abiotic hydrolysis blank.
4. 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)

VII. 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.

VIII. Centrifugation

1. Pipette 1 mL of supernatant into microcentrifuge tubes.
2. Cap and centrifuge at 14,000 rev min^{-1} for 1:45 minutes.

IX. Colorimetry

1. 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.

X. 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.

XI. 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 = 0.125

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*0.125 - 0.0351 = 0.001 mM

Convert sample concentration to activity:

$$Potential\ activity = \frac{\left(\frac{0.375mM * 1}{0.8988} - 0.068mM - 0.001mM\right) * 0.01L * \frac{1000\mu mol}{mmol}}{1g\ soil * 24hr}$$

$$= \mathbf{0.1451\ \mu mol\ pNA\ g^{-1}\ soil\ h^{-1}}$$

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:

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