

SOP: Enzyme assays (pNP)

Overview:

This standard operating procedure (SOP) describes the measurement of potential soil enzyme activities using *para*-nitrophenyl (pNP) linked substrates that releases *para*-nitrophenol (pNP) when hydrolyzed by soil enzymes. The method was originally reported by Tabatabai and Bremner (1969) and further modified in Tabatabai (1994), Margenot et al. (2018), and Daughtridge et al. (2021). Field moist soil samples (stored at $\approx 4^\circ\text{C}$ and hand-crumbled) or air-dried and ground to pass a $< 2\text{ mm}$ sieve are used.

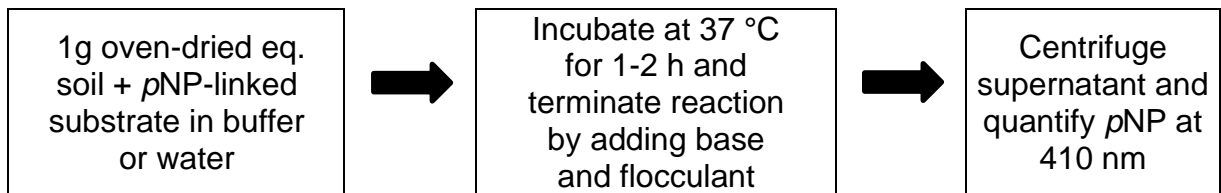
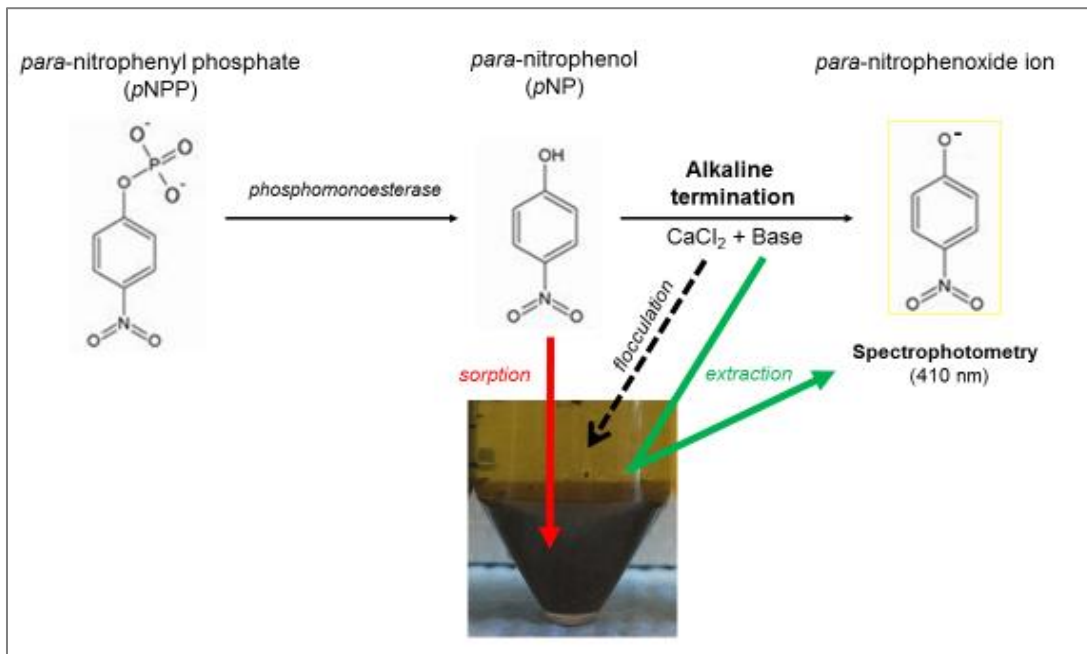


Figure shows how a pNP-linked substrate yields pNP during enzyme assay and how pNP interacts with the soil matrix.



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:

Eye Protection: Safety goggles

Body Protection: Lab coat

Hand Protection: Gloves

Particularly hazardous substances: *para*-nitrophenol (also called 4-nitrophenol) and hydrochloric acid

Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Sample preparation

- Analytical balance (two decimal places sensitivity)
- 50 mL centrifuge tube

Reagent preparation

- Analytical balance (at least three decimal places sensitivity)
- pH meter
- Pipettes & tips (100 μ L – 4 mL)
- 500 mL volumetric flask
- Boric acid
- Citric acid (monohydrate)
- Maleic acid
- NaOH
- HCl
- *p*NP-substrate
- THAM (also called TRIS, Trizma base)
- CaCl₂
- *p*NP (4-nitrophenol)
- 18.2M Ω -cm water

Enzyme assay

- Pipettes & tips capable of dispensing 100 μ L – 4 mL
- Water bath capable of maintaining 37 °C

Colorimetry (quantification of *p*NP)

- microcentrifuge and microcentrifuge tubes (1-2 mL)
- microplate spectrophotometer (absorbance measured at 410 nm)
- 96-well microplates

Detailed Procedure:

I. Sample and Blank Preparation

1. Measure 1.00 ± 0.02 g of oven-dried equivalent of soil into 50 mL centrifuge tube. Record exact weight of soil to at least 1/100th of one gram (1.XX g)
2. In addition to the soil samples, prepare blanks that are going to be treated the same way as samples (sections III to V).
 - i. **True blank** with no soil and no substrate. Accounts for background absorbance of matrix solution and also helps detect any contamination throughout assay.
 - In a 50 mL centrifuge tube, add 5 mL of water.
 - This may be used as 0 mM pNP standard for the calibration curve.
 - ii. **Abiotic hydrolysis of substrate:** Blank with same pNP-substrate solution as samples, but with no soil: accounts for abiotic hydrolysis of substrate during storage, incubation, and termination.
 - In a 50 mL centrifuge tube, add 5 mL of pNP-substrate solution.
 - iii. **pNP-recovery:** Blank with pNP solution (not pNP-substrate) and soil. Accounts for sorption/recovery of pNP as pNP can be bound to soil particle surface. Soils can vary in sorption capacity of pNP, and thus a priori testing of representative samples from the study site is recommended to check if recovery correction is necessary. The concentration of pNP to be spiked should be close to the amount your soil hydrolyzes.
 - In a 50 mL centrifuge tube, add 1.00 g of soil and 5 mL of pNP-stock solution (the stock solution may be diluted down before spiking to mimic amount of hydrolysis in soil samples; e.g., 1 mM).
 - iv. **DOM:** Blank with soil but without substrate. Accounts for dissolved organic matter (DOM) interference. This is critical when you see dark brown-black color in your terminated assay solutions even after centrifugation because this can contribute to absorbance at 410 nm that is not from pNP.
 - In a 50 mL centrifuge tube, add 1.00 g of soil and 5 mL of water.

Note: True blank and abiotic hydrolysis of substrate blank are required for every enzyme assay (e.g. per enzyme), whereas pNP-recovery and DOM blanks are soil-specific therefore may be run only once if running multiple enzymes on the same soils. Abiotic hydrolysis blank and DOM account for overestimation of activity due to absorbance that is not from soil enzyme activity, whereas pNP-recovery blank accounts for underestimation of activity. Detailed illustration of these blanks can be found in Daughtridge et al. (2021).

II. Reagent preparation

1. Modified universal buffer (MUB)

* This is not necessary if you use water as matrix instead of buffer. Buffer has been traditionally used to maintain reaction solution at assumed pH optima (for each enzyme) during assay. However, evidence suggests that buffer does not maintain the pH at target pH during assay (Li et al., 2021), and assumed pH optimum does not necessarily correspond to highest activity (Wade et al., 2021). Therefore, the use of water is suggested to avoid false assumption of pH optima and conduct assay in more natural condition (i.e., soil pH).

- i. MUB stock, as per Turner method (Turner, 2010)
 - Make solution of the following for a final volume of 500 mL using a volumetric flask
 - a. 6.3 g boric acid
 - b. 14.0 g citric acid (15.3 g for citric acid monohydrate)
 - c. 11.6 g maleic acid
 - d. 12.1 g THAM
 - e. 19.5 g NaOH
 - MUB stock keeps in fridge for ~2 weeks without risk
- ii. Dilute MUB stock 4x (1:3) with distilled water to make a working MUB solution
 - Use NaOH and HCl solutions (5-10 w/v or v/v%) to adjust the working MUB solution pH to target. See table below for target pH values (based on enzyme optima)

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

Abbr.	Enzyme	Cycle	Substrate	CAS Number	Molar weight (g)	Time (h)	[Subs] mM
BG	Betaglucosidase	C	pNP-β-D-Glucopyranoside	2492-87-7	301.25	1	10
NAG	Chitinase	C and N	pNP-N-acetyl- β -D-Glucosaminide OR pNP-2-acetamido-2-deoxy-β-D-glucopyranoside	3459-18-5	342.3	1	10
PME	Phosphomonoesterase	P	pNP Phosphate Disodium Salt Hexahydrate	4264-83-9/333338-18-4	371.14	1	20
PDE	Phosphodiesterase	P	Bis(4-Nitrophenyl) Phosphate	645-15-8	340.18	1	10
SUL	Sulfatase	S	pNP sulfate potassium salt	6217-68-1	257.26	1	10
CBH	Cellobiohydrolase	C	pNP β-D-cellobioside	3482-57-3	463.39	2	2

* Substrate concentration ([Subs]) may be well below saturation for many enzymes. It is recommended that K_m be determined for each soil sample or that a high [Subs] be used. See Margenot et al. 2018 for discussion.

2. pNP-substrate solution, made in buffer at final assay pH or in water
 - i. For traditional, operational assessments of enzyme activities, pNP-substrate solution of 0.010 M = 10 mM per g soil is used
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- Use 20 mM for P-cycling enzymes
- However, high concentrations of substrate achieve substrate saturation of an enzyme → better reflect activity differences at V_{\max}
 - a. Lower risk of false negatives
 - b. See Margenot et al. (2018) for details
- Most of the substrate solutions should be stable at 4 °C for ~ 1 week (Daughtridge et al., 2021)
- ii. Substrate concentration used can vary among studies; however, it is recommended to stick to a same substrate concentration throughout a study for comparability.
- iii. Note that this SOP describes single-point enzyme assay involving single substrate concentration. For kinetic characterizations, you need to vary substrate concentrations. See Margenot et al. 2018 SBB for example for phosphomonoesterase.
- iv. To determine the substrate mass (g) required for a given volume:

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

Example for BG:

Number of samples = 105

Volume of substrate solution per sample = 5 mL

Volume total = 525 mL

$$\text{Substrate mass (g)} = 0.525 \text{ (L)} \times 0.01 \left(\frac{\text{mol}}{\text{L}} \right) \times 301.25 \left(\frac{\text{g}}{\text{mol}} \right)$$

$$\text{Substrate mass (g)} = 1.5816 \text{ g of pNP-}\beta\text{-D-Glucopyranoside substrate}$$

3. 0.1 M THAM at pH 12

- i. Prepare 0.1 M THAM and adjust the pH using NaOH and HCl solutions (5-10 w/v or v/v%) to yield final pH of 12.
 - Base is used to halt reaction and activate pNP chromophore via alkalization (see Fig. 1)
 - The base also helps extract pNP sorbed to soil particles
 - 0.5 M NaOH was originally used until evidence of its hydrolysis of substrates and thus potential overestimation of potential activities appeared. Although this can be corrected with your soil-free blank, abiotic hydrolysis may be higher than enzyme activity in some cases (Daughtridge et al., 2021). THAM is also preferred because NaOH can cause

precipitation of Ca due to high pH after termination of reaction

- ii. THAM is also called TRIS, Trizma base
4. 2.0 M CaCl₂
- i. Prepare 2.0 M CaCl₂ solution
 - It is used to flocculate soil after termination with base
 - 2.0 M is higher than traditional 0.5 M concentration. This is to avoid DOM co-extraction with alkaline termination, especially for high OM soils. See Margenot et al. (2018) for rationale.
5. pNP (4-nitrophenol) standards made in same matrix (buffer or water) as enzyme assays and terminated the same way as samples, for calibration curve for colorimetry.

Example Approach:

- i. Make **pNP-stock solution** by mixing 0.0348 g of *para*-Nitrophenol in 50mL of water to make 5 mM solution.
Note: If you have already made the solution for the pNP-recovery blank, then you do not need to remake this.
- ii. Dilute by adding 1mL stock solution (from i) with 7mL water in a new centrifuge tube.
- iii. Take 5mL from (ii) and transfer it to a new centrifuge tube, then add 1 mL CaCl₂ + 4mL THAM.
- iv. Dilute this solution (from iii) into the microcentrifuge tubes following steps below.
- v. Hand shake tubes, then pipette 200 µL to well plate and read at 410 nm.

Dilution	Volume of Standard (mL)	Volume of water (mL)	Concentration for curve (mM)
1x	0.50	0	0.3125
2x	0.25	0.25	0.1563
3x	0.25	0.50	0.1042
4x	0.25	0.75	0.0781
5x	0.25	1	0.0625
10x	0.1	0.9	0.0313
20x	0.25 (of the 10x)	0.25	0.0156

III. Reaction

1. Turn on the water bath in advance so that the temperature stabilizes at 37 °C. Add water if the water level is too low.
2. Uncap the 50 mL centrifuge tube (with weighed soils) and add 5 mL of pNP-linked substrate solution to the 50 mL centrifuge tube
3. Swirl the centrifuge tubes for 1 min. Place plastic wrap on top of the tubes to avoid evaporation during incubation. Then incubate the tubes at 37 °C for 1 or 2 hr (depends on which enzyme being evaluated) using the water

bath. To keep the tubes inside the water bath, place weight (e.g., tray + flasks filled with water) on top of the tubes.

IV. Alkalization and centrifugation

1. At the end of incubation, take centrifuge tubes out of the water bath and terminate reaction by adding 4 mL of 0.1 M THAM (reactions of soils and working standards should turn yellow in the presence of *p*NP)
2. Add 1 mL 2.0 M CaCl₂ to flocculate; let sit ~5 min or until clear aliquot is evident
3. Transfer 1 mL of supernatant of resting reaction solution into a microcentrifuge tube and spin down (14,000 rpm for 1 min 45 s) or until a clear supernatant is obtained.

V. Colorimetry

1. Making sure to not disturb sedimented pellet at bottom of microcentrifuge tube, transfer 200 μ L to the 96-well microplate. For some enzymes (in particular phosphomonoesterase) and/or some soils, you may need to dilute supernatant using nano-pure water to achieve an absorbance value in the linear range of the calibration curve. You can dilute the sample directly in well plate (e.g., adding 100 μ L of sample + 100 μ L of water). Make note of any dilutions made for calculation of potential enzyme activity.
2. Turn on the microplate spectrophotometer to warm up, set absorbance reading at 410 nm (literature uses absorbance at 405-420 nm to quantify *p*NP).
3. Measure absorbance at 410 nm of the calibration standards, soil reaction extracts, and blanks. Make sure that the standard curve is linear (typically $R^2 > 0.99$), and absorbance of your samples and blanks are within the linear range of constructed standard curve.

VI. Clean up

1. Collect all solutions containing *p*NP or *p*NP-linked substrate (and buffer if used) into chemical waste bottle, clearly labelled with contents and their concentrations. Request pick up when the bottle is almost full. Clean up any spills with absorptive tissues and soap water immediately as needed.

VII. Calculations

Potential enzyme activities are usually expressed in units of $\mu\text{mol } p\text{NP g}^{-1} \text{ soil hr}^{-1}$. You may also see these reported as $\text{mg } p\text{NP g}^{-1} \text{ soil hr}^{-1}$, but this is inaccurate because the mass of *p*NP is arbitrary when considering the capacity of an enzyme to hydrolyze real substrates. Note that enzyme activities are rates of substrate transformation but is “potential” because substrate concentrations in assays are likely much higher than in situ conditions (actual activities). However, this method has been widely applied and “potential” activity has been shown to detect differences in factors such as land use and management.

1. Convert absorbance reading to concentration (mM) based on the calibration curve you obtained from pNP standards
2. If you have diluted the samples, multiply the concentration by dilution factor
3. If you have done the pNP-recovery blank, divide the concentration from step 2 by the recovery ratio to correct for the sorption. Recovery calculated as ratio of mole of pNP recovered (concentration of recovery blank from step 2 minus concentration of DOM blank, then multiplied by final volume) to mole of pNP spiked (concentration of spike solution multiplied by volume spiked).

pNP recovery ratio

$$= \frac{(\text{Concentration of pNP recovery blank (mM)} - \text{Concentration of DOM (mM)}) \times 0.01L}{\text{Amount of pNP spiked (mmol)}}$$

Example:

Concentration of pNP-recovery blank (after correcting for dilution, if diluted) = 0.499 mM

Concentration of DOM blank = 0.002 mM

Volume of pNP solution spiked = 5 mL = 0.005 L

Concentration of pNP spiked = 1 mM

$$pNP - \text{recovery ratio} = \frac{(0.499 - 0.002) \times 0.01}{0.005 \times 1} = 0.994$$

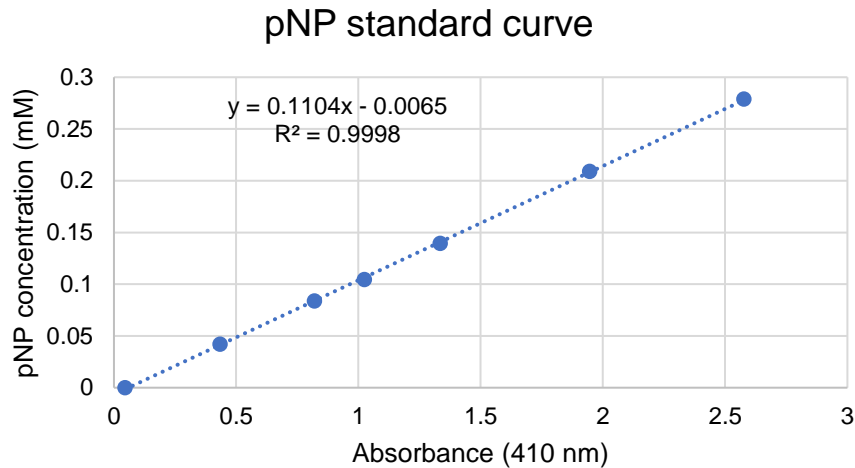
4. Convert the hydrolysis blank absorbance to concentration (mM) using the same standard curve as step 1 and subtract the resulting concentration from corresponding sample concentrations calculated in step 3 (make sure that initial substrate concentration of blanks and samples correspond to each other)
5. If you have done the DOM blank, convert the DOM blank absorbance to concentration (mM) using the same standard curve as step 1 and subtract the resulting concentration from sample concentration with same soil used
6. Regardless of whether you have done the above correction (you should) or not, convert the concentration (mM) to the amount (mmol) of pNP hydrolyzed in the final solution by multiplying the concentration with the volume of your final solution in L after termination (10 mL = 0.01L). Then, multiply the value by 1000 to convert to μmol .
7. Divide the μmol pNP hydrolyzed by the amount of oven-dry soil (g) and duration of incubation (hr) to obtain $\mu\text{mol pNP g}^{-1} \text{ soil hr}^{-1}$

Example calculation:

Given

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Sample Absorbance = 1.021
 Dilution = 2 (for both sample and blank)
 Standard curve $y = 0.1104x - 0.0065$
 p NP recovery ratio = 0.973
 Hydrolysis blank absorbance = 0.115
 DOM blank absorbance = 0.09



Convert absorbance to concentration
 Sample conc. = $0.1104 \times 1.021 - 0.0065 = 0.106 \text{ mM}$
 Hydrolysis blank conc. = $0.1104 \times 0.115 - 0.0065 = 0.00484 \text{ mM}$
 DOM blank conc. = $0.1104 \times 0.09 - 0.0065 = 0.00203 \text{ mM}$

Convert sample concentration to activity (with correction for DOM and sorption)

$$\begin{aligned}
 \text{Potential activity} &= \frac{\left(\frac{0.106 \text{ mM} * 2}{0.973} - 0.00484 \text{ mM} * 2 - 0.00203 \text{ mM} * 2 \right) * 0.01 \text{ L} * \frac{1000 \mu\text{mol}}{\text{mmol}}}{1 \text{ g soil} * 1 \text{ hr}} \\
 &= 2.05 \mu\text{mol } p\text{NP g}^{-1} \text{ soil h}^{-1}
 \end{aligned}$$

Convert sample concentration to activity (without correction for DOM and sorption)

$$\begin{aligned}
 \text{Potential activity} &= \frac{(0.106 * 2 - 0.00484 * 2) * 0.01 * 1000}{1 * 1} \\
 &= 2.02 \mu\text{mol } p\text{NP g}^{-1} \text{ soil h}^{-1}
 \end{aligned}$$

References:

Daughtridge, R.C., Nakayama, Y., Margenot, A.J., 2021. Sources of abiotic hydrolysis of chromogenic substrates in soil enzyme assays: Storage, termination, and incubation. *Soil Biology and Biochemistry* 158, 108245.

Li, C., Wade, J., Margenot, A.J., 2021. Modified universal buffer does not necessarily maintain soil enzyme assay pH. *Biology and fertility of soils* 57, 869-872.

Margenot, A.J., Nakayama, Y., Parikh, S.J., 2018. Methodological recommendations for optimizing assays of enzyme activities in soil samples. *Soil Biology and Biochemistry* 125, 350-360.

Tabatabai, M., 1994. Soil enzymes. *Methods of soil analysis: part 2—microbiological and biochemical properties*, 775-833.

Tabatabai, M., Bremner, J., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry* 1, 301-307.

Wade, J., Li, C., Vollbracht, K., Hooper, D.G., Wills, S.A., Margenot, A.J., 2021. Prescribed pH for soil β -glucosidase and phosphomonoesterase do not reflect pH optima. *Geoderma* 401, 115161.

Suggested reading:

1. 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>

2. 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>

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Questions can be directed to Andrew Margenot at margenot@illinois.edu