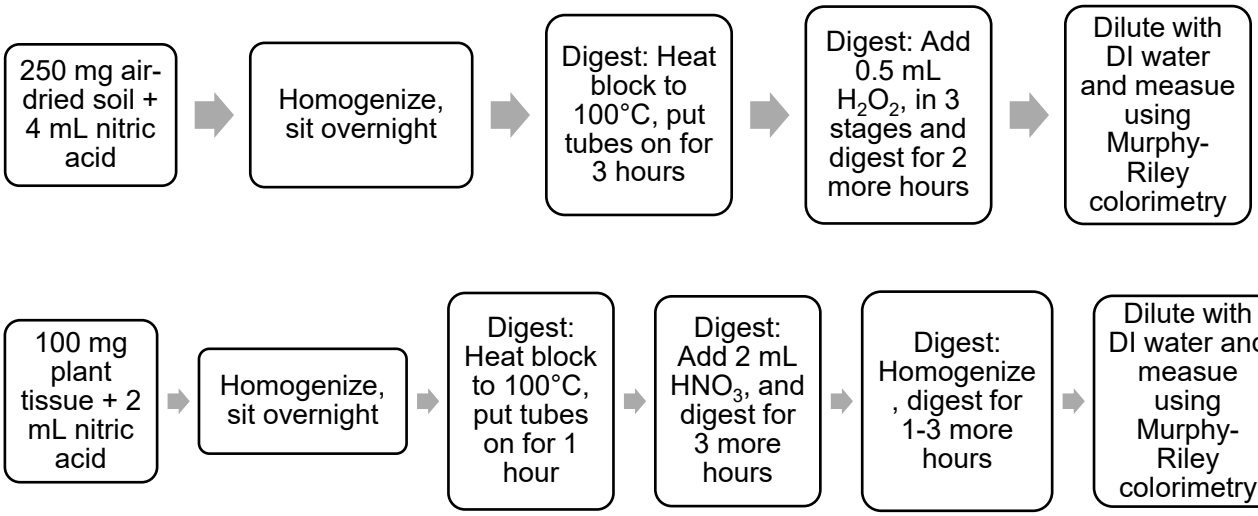
SOP: **Soil or Plant Material Total P Digestion**

Overview:

This standard operating procedure (SOP) describes a protocol commonly used for quantifying total P in soils or plant material. The method is based on EPA method 3050B (USEPA 1996) and was modified by Chongyang Li and Devin Rippner (Li et al 2021) from White Jr and Douthit (1985). Samples should be air-dried and ground to a powder (<0.5 mm) for best results. Note that lithium borate fusion (Soils Lab, 2023) is the preferred method for measuring total P in soils as the nitric-peroxide wet digestion does not fully break down the soil structure and therefore does not achieve a complete recovery.



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: Acid resistant nitrile gloves
Particularly hazardous substances: Concentrated nitric acid, sulfuric acid, and hydrogen peroxide should be handled in the fume hood. Make sure to check Safety Data Sheet if unsure about how to handle these chemicals.
Specific details on these substances are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Sample preparation

- Analytical balance (two decimal places sensitivity)
- 15 mL centrifuge tube – must be polypropylene centrifuge tubes (Falcon brand)

Reagent preparation

- Concentrated trace metal grade nitric acid (67-70%)
- Hydrogen peroxide (30%)
- Sulfuric acid (98%)
- Commercial P standard (1000 mg P/L)
- Ammonium molybdate
- Antimony potassium tartrate
- Ascorbic acid

Digestion

- 15 mL centrifuge tubes
- Vortex mixer or horizontal shaker
- Heat block (up to 100°C)
- Pipette and tips (125 - 250 µL)
- Concentrated trace metal grade nitric acid (67-70%)
- Hydrogen peroxide (30%)

Colorimetry

- 96 well microplates
- Microplate spectrophotometer
  - Note: this SOP's colorimetry procedures are based on microplates, but can be scaled for cuvettes while keeping same ratio of reagents and extracts
- Pipette and tips (100-1000 µL)
- 10% NaOH
- 4-nitrophenol or pH paper

Detailed Procedure:

I. **Sample Preparation**
   1. Measure 250±10 mg of air-dried soil or 100 mg plant material into 15 mL graduated centrifuge tube. If the material is rich in P (>1000 ppm), reduce the material weight to 100 mg. Polypropylene Falcon tube is necessary to avoid melting and burning issues with the heat block (i.e., not polystyrene or polyethylene). Record weight of soil or plant material to the nearest 1/100th of a gram.
   2. Prepare at least two empty 15 mL centrifuge tubes for blanks (no soil or plant material, but treated the same way as samples, for quality control)

II. **Reagent preparation**
   1. No preparation for nitric acid and hydrogen peroxide.

2. Standards
    i. Calibration standards (ranging from 0 – 40 mg P/L) need to be made in each extracting solution and undergo the same neutralization process as the samples. Dilute commercial standard (1000 mg P/L) in each extracting solution and sequentially dilute them to make calibration standards that cover the concentration you are expecting for your soil samples. It is essential to use the same extracting solution because molybdate colorimetry of P is sensitive to pH (color development and intensity, precipitation).
    ii. To prepare standards from a 1000 ppm P stock solution: Create a 100 ppm aqueous stock solution by combining 2 mL 1000 ppm stock with 18 mL nanopure water. Then, combine the new 100 ppm stock with nitric acid and DI water in 15 mL centrifuge tubes to produce a range of standards in a matrix that reflects the 10 mL digestate. For example:

Standard:	0 ppm	2.5 ppm	5 ppm	10 ppm	15 ppm	20 ppm	30 ppm	40 ppm
100 ppm stock (ml)	0	0.25	0.5	1	1.5	2	3	4
Nitric acid (~70%) (ml)	4	4	4	4	4	4	4	4
DI water (ml)	6	5.75	5.5	5	4.5	4	3	2

1. Golden Rule: Treat standards the same as samples. This goes for the working standards being made up in the same solution as extracts and undergo the same neutralization process. If colorimetry reactions are diluted to bring absorbance into linear range, do the same for standards, etc. **Make P standards in the same nitric acid solution**; dilute from 1000 mg P/L standard in S-23 fridge directly into the nitric acid solution used for the digestion.

3. Colorimetry reagents
    i. Murphy-Riley Solution A
        a. Dissolve 4.3 g ammonium molybdate in 400 mL of deionized water in a 1.000 L volumetric flask.
        b. Dissolve 0.40 g antimony potassium tartrate in 400 mL deionized water, then add to the ammonium molybdate solution in the volumetric flask from step a.
        c. Slowly and carefully, with stirring and while cooling in an ice bath, add 54 mL concentrated H_2SO_4.
        d. Allow to cool and make to 1000 mL in the volumetric flask with deionized water. Mix well and store in a dark bottle in a refrigerator. The reagent is stable for 4 weeks at 4°C.
    ii. Murphy-Riley Solution B

a. Ascorbic acid, 1%: Dissolve 1.00 g of ascorbic acid in 100 mL deionized water. Make a fresh solution daily as needed.

iii. Final Murphy-Riley (MR) reagent: combine 56 mL of solution B + 44 mL of solution A, and mix (should turn to light yellow color)

III. **A. Digestion - Soil**

1. Add 4 mL of concentrated trace metal grade nitric acid (67-70%) into the pre-weighed material and tightly cap the tube. Vortex or homogenize samples on a horizontal shaker for 15-30 seconds.
2. Allow the capped tubes to sit overnight at room temperature (in fume hood). Shake samples for another 15-30 seconds prior to adding to the heating block.
3. Preheat block digester to 100 °C in the fume hood. When the block is up to temperature, transfer the tubes in with the caps set aside for later use. Digest tubes for 3 hours or until the brown fumes, if any, stop forming during this process.
4. Carefully add 0.125 mL of trace metal grade H_2O_2 into the tube, drop by drop.
5. Repeat step 4 with another 0.125 mL H_2O_2.
6. Carefully add 0.250 mL of trace metal grade H_2O_2 into the tube, drop by drop. Digest for another 2 hours.
   NOTE: Adding hydrogen peroxide to the hot digests causes it to bubble up, so it is important to add just a drop or two at a time to avoid overflow. Furthermore, there should be at least 30 seconds between the first, second, and third addition to allow the effervescence to subside.
7. After 2 hours of digesting with hydrogen peroxide, the bubbling should be minimal and no brown fumes present. You can add up to 1 additional hour of heating if this is not the case. Pull tubes from block and dilute to 10 mL with 18.2 MΩ nanopure water while still warm. Homogenize and allow remaining sediment to settle overnight.
   NOTE: The digests may be cloudy or even dark after diluting to 10 ml; this is normal (see image 1 below). The presence of dark fumes is a more reliable indicator of the stage of the reaction than the color of the digest itself.
8. Pipette 1 mL out of the 10 mL solution from step 7 to do another 5x dilution using DI water. *Also dilute each of the P standards by a factor of 5 at this stage.*

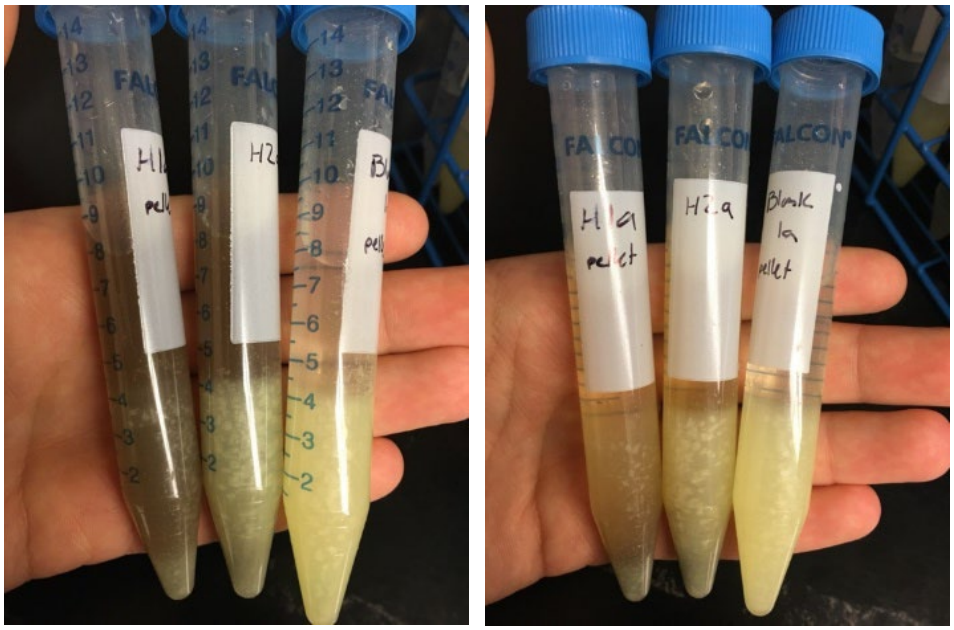


Image 1 Soil digests after diluting and homogenizing (left) and after being allowed to settle overnight (right). Note that the acid and heat has weakened the tubes and they cannot be centrifuged in this condition.

B. Digestion – Plant tissue

1. Add 100 mg plant tissue to 15 mL Falcon brand centrifuge tubes. (*Make sure to include digestion blanks and reference materials*.)
2. Add 2 mL of concentrated trace metal grade nitric acid (67-70%) and vortex for a few seconds.
3. Allow to sit overnight at ~25 °C. Vortex samples again prior to adding to the heating block.
4. Heat block to 100 °C (90 °C if using VWR falcon tubes) and digest for 1 hour with the caps off and set aside; if brown fumes form during this process, add 2 ml more HNO_3 and digest at 100 °C for another 3 hours. **Watch for bubbling when adding HNO_3, do not allow to bubble over.**
5. Remove samples from heat block, replace caps and vortex for a few seconds.
6. Remove caps and set aside. Put tubes back on block, digest at 100 °C for 1-3 hours (wait for bubbling and brown fumes to stop and a clear digest as an indicator of completion of digestion). **Watch for bubbling, do not allow to bubble over.**
7. When clear and bubbling has stopped, remove tubes from block and dilute to 10 ml as marked on the exterior of the centrifuge tubes.
8. Dilute 5-10x for colorimetric analysis.

IV. A. P colorimetry - Soil

1. Sample neutralization and colorimetry is performed directly in the well plates (standard disposable polyacrylamide or another cheap polymer). 96 well plates hold up to 350 µL.
    i. Test varying ratios of the diluted digestate and 10% NaOH to create a solution that is approximately neutral (pH 7) with a combined volume of 80 µL. A 1% aqueous solution of 4-nitrophenol may be used as a pH indicator as it is yellow in color but turns clear at a neutral pH.
    ii. Pipette samples, 10% NaOH, and Murphy-Riley reagent into well plates being sure to include standards, blanks, and replicates.
    iii. A ratio of 57 µL sample: 23 µL 10% NaOH : 80 µL MR reagent has worked for soils in the past.
2. After pipetting, set plates aside to develop for 15 minutes. Absorbance is measured on the plate reader at 882 nm.

B. P colorimetry – Plant tissue

1. First, determine how much of 10% NaOH solution is needed to neutralize the highly acidic digest
2. Pipette 500 µL of digest and add to cuvette; then, add 1 drop (5 µL) of 1% 4-nitrophenol solution as a pH color indicator. This will make the solution turn yellow when pH > 7.6
3. Add small volumes of 10% NaOH (50-100 µL is usually useful) to digest to gauge how much volume of 10% NaOH is needed to achieve pH 7.6. This will likely take several iterations. Enough base should be added to bring digest supernatant just below pH 7.6
4. Once the amount of 10% NaOH needed to neutralize the digest is determined (should be done for each tissue type, but not necessary for every single sample), perform P colorimetry
    i. First, add 10% NaOH solution to cuvette. Then, add 500 µL digest supernatant. Finally, add 700 µL Murphey-Riley solution. Note these are suggested volumes; actual volume can be changed by scaling while maintaining the ratios
    ii. Add DI water if need to dilute if strong absorbance is observed. May need to scale back how much of digest supernatant is reacted if samples are extremely high in P (above standard curve linearity)
5. After pipetting, set cuvettes aside to develop for 15-20 minutes. Absorbance is measured at A882.

V. Clean up

1. Make sure to turn off and unplug the heat block.
2. Collect solutions (except those that can go into drain with copious amount of water; check DRS for further information) 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. For spilled dilute acids, immediately neutralize with sodium bicarbonate and then clean up.

VI. **Calculations**

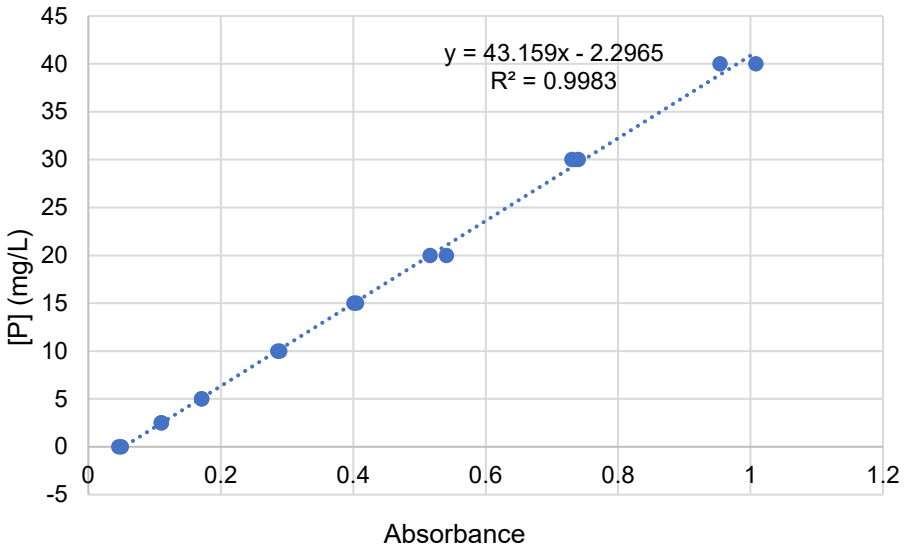
Measurement of P fractions is usually expressed in units of mg P kg^{-1} soil.

1. Convert raw absorbance to concentration (mg P L^{-1}) using calibration curve (as noted previously, calibration standards should be treated exactly the same way as samples, so the absorbance can be directly converted to concentrations in the extract). Multiply the concentration by dilution factor if diluted.
2. Multiply the concentration by the digestate volume (e.g., 10 mL = 0.010 L) and divide by soil mass (0.250 g = 0.00025 kg) to yield concentration in mg P kg^{-1} soil.

Example calculation:

Absorbance = 0.315
Dilution = 1
Calibration curve: y = 43.159x – 2.2965
Extraction volume = 0.010 L
Soil mass = 0.00025 kg

Concentration in extract = 43.159 * (0.315) – 2.2965 = 11.3 mg L^{-1}
Concentration in soil basis = 11.3 mg L^{-1} * 0.010 L / 0.00025 kg = 452 mg P kg^{-1} soil



A note on total P determination in soils:

A number of options are available when it comes to selecting a method for total P determination in soils. This nitric-peroxide block digestion typically captures 92-96% of P, depending on soil type, and is suitable for most laboratory setups. Similarly effective approaches include the sulfuric-peroxide digestion (Parkinson and Allen 1975), perchloric acid digestion (Olsen and Sommers 1982), and sodium hypobromite-sodium

hydroxide alkaline oxidation method (Dick and Tabatabai 1977). Though widely used, *we recommend against using these wet acid digestions methods for soils due to their incomplete recovery*, which varies with soil type. Hydrofluoric acid digestion may be used to obtain a complete recovery (Bowman 1988). However, HF must be used with extreme caution since a relatively small amount of skin contact can lead to fluoride poisoning and hypocalcemia. Lithium borate fusion, which utilizes inexpensive graphite crucibles and a benchtop muffle furnace, is the preferred method for total P analysis. A comparison of P recoveries from a nitric-peroxide wet digestion and lithium metaborate fusion are presented below:

Sample no.	1	2	3	4	5	6	7	8	9	10
nitric-peroxide (mg kg^{-1} P)	390	468	273	388	652	564	455	431	296	426
lithium borate (mg kg^{-1} P)	474	488	403	470	684	649	571	532	457	491

In this instance, lithium borate fusion recovered 20% more P than the nitric-peroxide digestion, on average.

References:

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Li, C., Rippner, D.A., Manavalan, L.P. *et al.* Evaluation of Bacillus seed coatings on soybean phosphorus uptake in an oxisol fertilized with ^{32}P-labeled hydroxyapatite. *Plant Soil* **464,** 273–287 (2021). https://doi.org/10.1007/s11104-021-04941-w

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Parkinson, J.A. and Allen, S.E. 1975. A wet oxidation procedure suitable for the determination of nitrogen and mineral nutrients in biological material. Soil. Sci. Plant Anal. 6:1-11.

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Thomas R White, Jr, Garnett E Douthit, Use of Microwave Oven and Nitric Acid-Hydrogen Peroxide Digestion to Prepare Botanical Materials for Elemental Analysis by Inductively Coupled Argon Plasma Emission Spectroscopy, *Journal of Association of Official Analytical Chemists*, Volume 68, Issue 4, 1 July 1985, Pages 766–769, https://doi.org/10.1093/jaoac/68.4.766

Suggested reading:

Church, C., Spargo, J., and Fishel, S. 2017. Strong acid extraction methods for “total phosphorus” in soils: EPA method 3050b and EPA method 3051. Agric. Environ. Lett. 2:160037.

Citation:

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