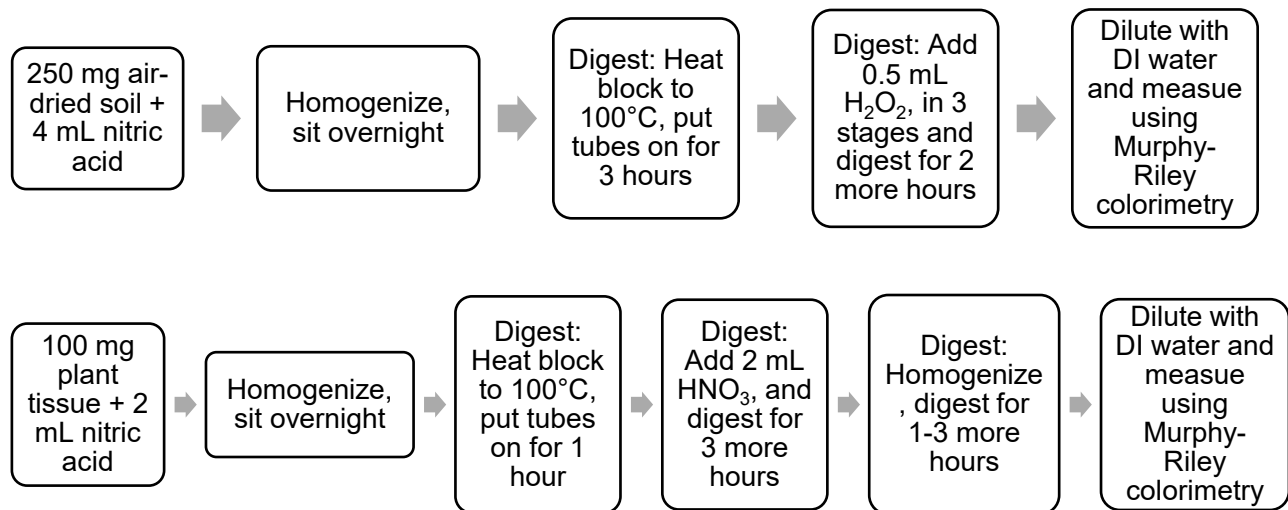


SOP: Soil or Plant Material Total P Digestion

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.

Several other methods may be used for total P determination. Techniques involving sodium carbonate or lithium metaborate fusion are extremely reliable but incur high upfront costs, particularly if a high throughput is desired (Kowalenko and Babuin 2014). Due to its ability to solubilize silicates, hydrofluoric acid digestions also provide a near-perfect 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. This nitric-peroxide block digestion method typically captures 92-96% of fusion- or HF-recoverable 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).



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 into 15 mL graduated centrifuge tube. If the material is rich in P (>1000 ppm), reduce the material weight to 100

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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

- iii. 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.

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 beaker.
- c. Slowly and carefully, with stirring and while cooling in an ice bath, add 54 mL conc. H₂SO₄.

- 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 H₂O₂ into the tube, drop by drop.
5. Repeat step 4 with another 0.125 mL H₂O₂.
6. Repeat step 4 for the third and final time, this time with 0.250 mL H₂O₂. 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 10 to do another 5x dilution using DI water. Also dilute each of the P standards by a factor of 5 at this stage.

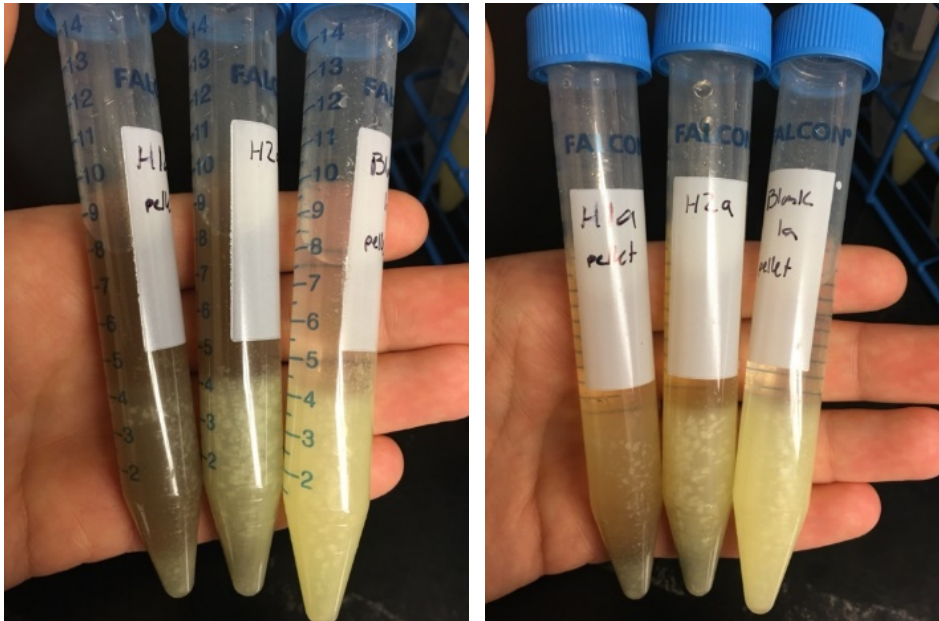


Image 1 Soil digests after diluting and homogenizing (left) and after being allowed to settle overnight (right). Note that the acid and heat has weakened the tubes and they cannot be centrifuged in this condition.

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.
3. Allow to sit overnight at ~25 °C. Vortex samples again prior to adding to the block.
4. Heat block to 100 °C (90 °C if using VWR falcon tubes) and digest for 1 hour; if brown fumes form during this process, add 2 ml more HNO₃ and digest at 100 °C and digest for another 3 hours.
5. **Watch for bubbling, do not allow to bubble over. Vortex.**
6. 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. Vortex repeatedly.**
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 with a combined volume of 80 μ L. A 1% aqueous solution of 4-nitrophenol may be used as a pH indicator.
 - ii. Pipette samples, NaOH, and Murphy-Riley reagent into well plates being sure to include standards, blanks, and replicates.
 - iii. A ratio of 57 μ L sample: 23 μ L 10% NaOH : 80 μ L MR reagent has worked for soils in the past.
2. After pipetting, set plates aside to develop for 15 minutes. Absorbance is measured at A882.

B. P colorimetry – Plant tissue

1. First, determine how much of 10% NaOH solution is needed to neutralize the highly acidic digest
2. Remove 500 μ L of digest and add to cuvette; then, add 1 drop (5 μ L) 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 (50-100 μ L is usually useful) to digest to gauge how much volume of 10% NaOH achieves 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 NaOH solution to cuvette. Then, add 500 μ L digest supernatant. Finally, add 700 μ L Murphey-Riley solution. Note these are suggested volumes; actual volume can be changed by scaling while maintaining 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. 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. Upper P standard should be 10-20 mg/L.
6. 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

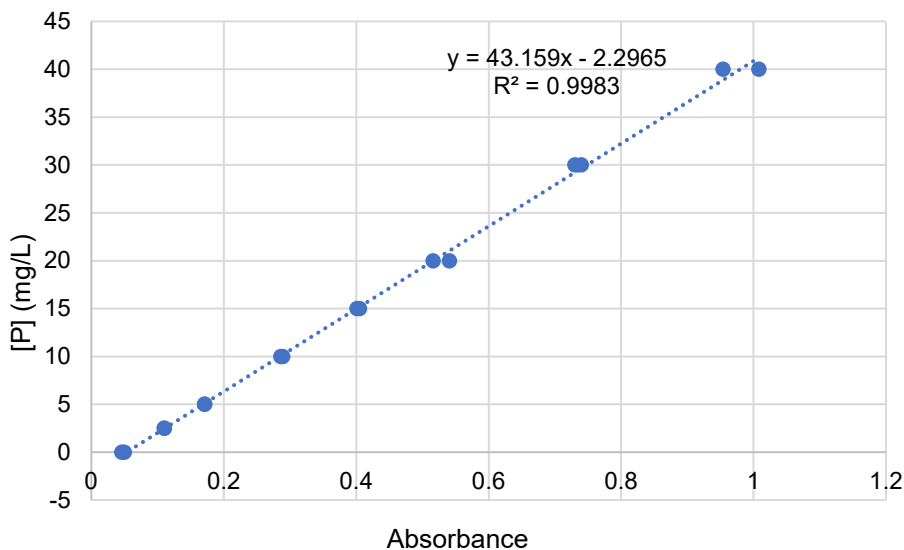
Calibration curve: $y = 43.159x - 2.2965$

Extraction volume = 0.010 L

Soil mass = 0.00025 kg

Concentration in extract = $43.159 * (0.315) - 2.2965 = 11.3 \text{ mg L}^{-1}$

Concentration in soil basis = $11.3 \text{ mg L}^{-1} * 0.010 \text{ L} / 0.00025 \text{ kg} = 452 \text{ mg P kg}^{-1} \text{ soil}$



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