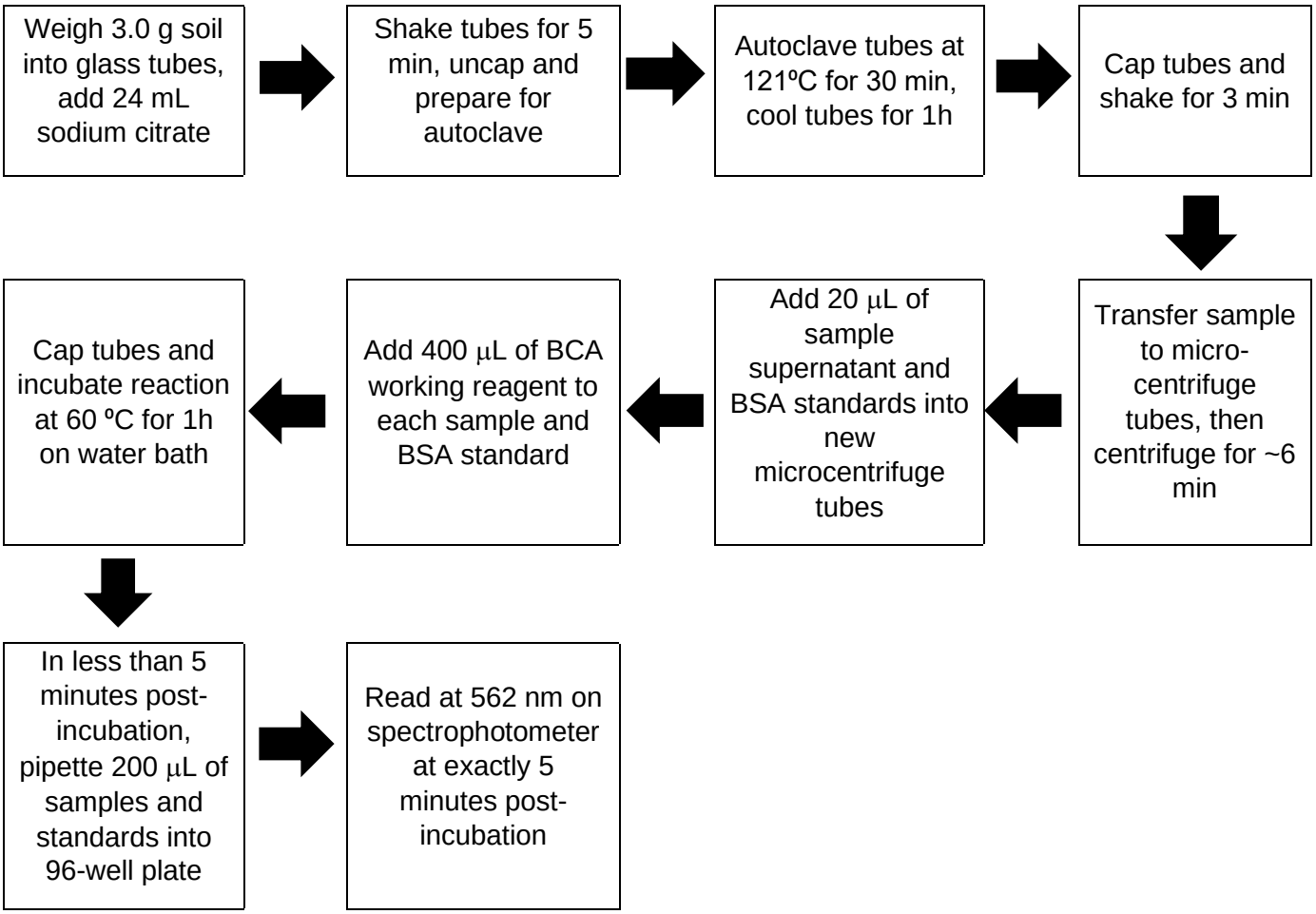
SOP: **Autoclave Citrate Extractable (ACE) Protein**

Overview:

This standard operating procedure (SOP) describes a protocol for determination of autoclave-citrate extractable (ACE) soil protein, originally outlined by Hurisso et al. (2018) using procedures reported to extract proteins from soil and fungi (Keen & Legrand, 1980; Wright & Upadhyaya 1996), and modified by Soils Lab UIUC in 2023. The calculation method to determine the mass of protein in each sample was also originally reported by Hurisso et al. (2018) and modified by Soils Lab UIUC in 2023. Autoclave-citrate extractable protein may be used as a rapid indicator of the organic nitrogen pool. Soils that are air-dried and ground to < 2 mm are always used. Key instruments and consumables include a sodium citrate stock solution, analytical balance, pH meter, shaker, autoclave, autoclave safe glass tubes and plastic tubs, caps for glass tubes, aluminum foil, microcentrifuge and microcentrifuge tubes, adjustable pipettes and pipette tips, water bath, spectrophotometer, and 96-well plates.



Safety:

All standard safety protocols and online safety training via UIUC Division of Research Safety (DRS) are required.
Personal protection equipment (PPE) for this procedure include:
Eye Protection: Safety goggles
Body Protection: A laboratory coat is recommended
Hand Protection: Disposable nitrile gloves when manipulating samples/reagents in lab. Heat protective gloves (capable of holding >121ºC) when taking samples out of the autoclave.
Particularly hazardous substances: Citric acid

- ✓ Wash hands thoroughly after handling
- ✓ Wear protective gloves/clothing/eye protection/face protection
- ✓ Do not eat, drink, or smoke when using this product
- ✓ IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present. Continue rinsing. If eye irritation persists, seek medical attention.

Specific details on this substance are incorporated in the **Detailed Procedure** below.

Instrumentation & Consumables:

Sodium Citrate Stock Solution

- Tribasic sodium citrate dihydrate ($C_6H_5Na_3O_7$, MW = 294.1 g mol^{-1}) (Organics cabinet)
- Citric acid ($C_6H_8O_7$, MW = 192.1 g mol^{-1}) (Organics cabinet)
- 20 L carboy
- 18.2MΩ cm^{-1} water source (or DI water source)
- 100 mL volumetric flask
- 1 L Erlenmeyer flask
- Stir bar
- Stir plate
- pH meter

Extraction and Clarification

- Analytical balance
- Autoclave capable of reaching 121 ºC for 30 min
- Autoclave safe glass tubes with fitting caps
- Autoclave safe rectangular plastic tub to hold tubs of samples
- Autoclave safe Nalgene plastic tubs that will hold glass tubes
- Aluminum foil
- Autoclave tape
- Heat resistant gloves
- Styrofoam 50 mL falcon tube racks
- Dispensette set to dispense 24 mL
- Plastic jug compatible with dispensette
- Shaker
- 1000 µL pipette and tips

- Microcentrifuge
- 1.5 mL microcentrifuge tubes
- Hollow microcentrifuge tube racks (S-27, marked for ACE protein)

Sample Reaction and Colorimetry

- Clear, polystyrene 96-well plate
- Thermo Scientific™ Pierce™ BCA Protein Assay Kit (1 L kit = Fisher Scientific # PI23225)
    - BCA reagents A & B
    - 2000 μg mL BSA standard solution
- One 50 mL falcon tube
- 10 mL pipette and tips
- 1000 μL pipette and tips
- 200 μL pipette and tips
- 20 μL pipette and tips
- Water bath capable of maintaining 60 ºC for 1 h
- Spectrophotometer

Detailed Procedure:

I. 20 *mM* Sodium Citrate Stock Solution (makes 20 L) Preparation

Note: This step may be done in advance, and the stock solution is stable at room temperature indefinitely. 20 L will accommodate approximately 800 samples. Once the pH is adjusted, no further adjustments to the pH are necessary.

1. Prepare 100 mL of 1 M citric acid by weighing 19.2 g citric acid, placing into a 100 mL volumetric flask, and adding 18.2MΩ cm^{-1} water up to 100 mL mark. A small amount of this citric acid solution will be used to adjust the pH of the stock solution to 7.0.
2. Weigh 117. 64 g of tribasic sodium citrate dihydrate and place into a 1 L Erlenmeyer flask. Place flask onto the stir plate, add the stir bar and approximately 500 mL of 18.2MΩ cm^{-1} water, and stir until sodium citrate dihydrate is dissolved completely.
3. Pour the sodium citrate dihydrate solution into the 20 L carboy, rise the Erlenmeyer flask with 18.2MΩ cm^{-1} water and pour into the carboy.
4. Fill the 20 L carboy with 18.2MΩ cm^{-1} water until it reaches the 20 L mark, and mix the solution by shaking the carboy, or use a stir plate if a large enough one is accessible.
5. Adjust the pH of the sodium citrate solution to a target of 7.0 using the citric acid solution. Since the solution is weakly buffered, only add a small amount (~1 mL) of the citric acid solution at a time, mixing the entire 20 L solution each time.

II. Standard Preparation

Eight solution standards (2000, 1500, 1000, 750, 500, 250, 125, 0 μg mL) are prepared from the bovine serum albumin (BSA) standard solution, which is a small glass vial found in the BCA Protein Assay Kit. The standard solution in the vial is 2000 μg mL and can be opened by breaking the vial at the white line towards the top. A pre-diluted set may be purchased from Fisher Scientific (# PI23208), but the chart below details a dilution process using the 2000 μg mL BSA standard solution included in the protein assay kit.

Standard Stock solutions: Use the table below to prepare standard stock solutions in 1.5 mL microcentrifuge tubes.

BSA Standard Solution concentration	Volume of BSA solution	Volume of sodium citrate buffer
2000 μg mL	n/a (leftover)	0 mL
1500 μg mL	0.450 mL	0.150 mL
1000 μg mL	0.300 mL	0.300 mL
750 μg mL	0.300 mL of 1500 μg mL	0.300 mL
500 μg mL	0.300 mL of 1000 μg mL	0.300 mL
250 μg mL	0.300 mL of 500 μg mL	0.300 mL
125 μg mL	0.300 mL of 250 μg mL	0.300 mL
0 μg mL	0 mL	0.500 mL

1. Label one microcentrifuge tube for each concentration of standard solution.
2. Using the chart above, dilute from 2000 μg mL until each concentration has been made.

III. Sample preparation and autoclave sterilization

1. Label one autoclave safe glass extraction tube for each sample, replications of samples are not necessary. Black sharpie may be used to label tubes, but it is recommended to label each tube at least twice in case the label is compromised during the autoclave cycle.
2. Weigh 3.0 g (± 0.05 g) of air-dried soil into each tube. Steps 1 and 2 may be done in advance.
3. Pour sodium citrate stock solution into plastic jug, attach dispensette to jug and adjust to dispense 24 mL of sodium citrate.

4. Add 24 mL of the 20 *mM* sodium citrate (pH = 7.0) stock solution to each glass tube containing the 3.0 g of soil.
5. Cap tubes tightly.
6. Place glass tubes in Styrofoam 50 mL Falcon tube holders (or anything that will hold the glass tubes on the shaker without breaking them) and shake for 5 min on the “low” setting at 180 oscillations per minute.
7. After 5 min, remove glass tubes from the shaker. Invert tubes to avoid soil clinging to caps or sides of the tubes.
8. Remove and rinse caps with water so they may be used later. Any soil or solution left on caps will compromise the seal later when the glass tubes need to be capped and shaken again after they are autoclaved.
9. Cover each tube with a small square of aluminum foil. This is to ensure that each tube is covered, without being sealed. **If tubes are sealed with caps they will explode in the autoclave.**
10. Once tubes are covered, place into autoclave safe Nalgene plastic tubs so that each tube is standing upright and supported in case they shake or move during the autoclave cycle. Autoclave tape or an autoclave safe material may be used to secure tubes.
11. Place a small amount of autoclave tape somewhere on the Nalgene tubs to ensure that 121 ºC was reached during the cycle. The tape should change color if the temperature has been reached; if the tape does not change color then the cycle will need to be re-run.
12. Place Nalgene tubs filled with glass tubes into another autoclave safe tub that will catch any spillover or melting. These tubs can typically be found in the room where autoclaves are, and this is a safety measure to protect the autoclave equipment from unintentional spillover, melting, or other issues.
13. Set autoclave to reach 121 ºC for 30 min. Place tub containing all samples into autoclave and run cycle.
14. Once cycle is finished, remove the tub using heat resistant gloves.
15. If samples have spilled over significantly into the plastic tubs, the samples that have spilled over should be re-run.
16. Set aside for approximately 1 h, or until glass tubes have reached room temperature.

Note: Autoclaves can be found in rooms M-427 and M-529 of Turner Hall and are available for all the CPSC department. To schedule autoclave time, sign into the autoclave log sheet next to each autoclave. If the autoclave is in use, refer to the person that is using it (can be found on the user information in the autoclave log sheet) to check if they are planning to run other batches, and/or if you could take turns to use it, prior to scheduling your autoclave time.

IV. Sample extraction and clarification

1. Label a 1.5 mL microcentrifuge tube for each extraction sample.
2. Once glass tubes have reached room temperature and can be handled safely, place clean caps tightly onto each glass tube.

3. Place tubes into Styrofoam racks, place onto shaker and shake for 3 minutes on the “low” setting at 180 oscillations per minute.
4. After they have been shaken, invert tubes to ensure no soil is clinging to cap or sides of the glass tube.
5. Remove caps.
6. Using a 1000 μL (1.0 mL) pipette, transfer 1.0 mL of each sample from the glass tubes into the labeled 1.5 mL microcentrifuge tubes. Use a new and clean pipette tip for each sample.
7. Close the microcentrifuge tubes and centrifuge for approximately 6 minutes at 17,968 RCF. Centrifuge samples until the soil has clearly separated to the bottom. The supernatant solution will likely be tinted in the color of the soil samples, but clear.

V. Reagent preparation for sample reaction and colorimetry

1. Turn on the water bath and set to 60 ºC.
2. Use one 50 mL Falcon tube to prepare the BCA working reagent. The working reagent is a 50:1 mixture of reagent A and reagent B. Using a 10 mL pipette, mix 25 mL of reagent A with 500 μL of reagent B using the 1000 μL pipette. This 25.5 mL solution makes enough for ~60 samples and standards. Cap the Falcon tube and invert several times to mix the working reagent solution.

VI. Sample reaction and colorimetry

1. Pipette 20 μL of the supernatant of each sample into new microcentrifuge tubes, taking care not to disturb the soil pellet at the bottom.
2. Pipette 20 μL of each of the prepared BSA standard concentrations into new microcentrifuge tubes as well.
3. Pipette 400 μL of the BCA working reagent into each microcentrifuge tube containing samples and standards.
4. Cap the microcentrifuge tubes and place on the water bath making sure that the portion of the tube that contains the supernatant is submerged (do NOT fully submerge the tube to avoid water going in). To keep the tubes inside the water bath, place weight (e.g., tray + flasks filled with water) on top of the tubes.
5. Incubate at 60 ºC undisturbed for 1 h.
6. A few minutes before the end of the incubation period, turn on the spectrophotometer and set the plate reader program (Gen5, at 562 nm).
7. After incubation, remove the tubes from the water bath and quickly pipette 200 μL of samples and standards into a 96-well plate. Cover with lid. **The samples must be read at exactly 5 minutes after the end of the incubation period**, so working in batches is recommended (e.g. at the maximum amount of samples that one can pipette into the well-plate in less than 5 minutes).

Note: If more than one batch, standard samples must be included in each batch (e.g. a whole new set of standards must be incubated and transferred to the well-plate along with the samples of the respective batch).

8. At exactly 5 minutes, remove the well plate lid and read at 562 nm.
9. Export the file to Excel and create the standard curve and linear equation using the concentrations (0, 125, 250, 500, 750, 1000, 1500, 2000) and absorbances of each concentration to make sure the R^2 of the linear equation is > 0.97.

VII. Calculations

The amount of protein extracted from the soil in each sample, expressed as g Protein kg^{-1} soil or g ACE-protein kg^{-1} soil, is calculated using the method described below. The equation results in total mass of extracted protein in μg mg^{-1} soil, which is equivalent to g ACE-protein kg^{-1} soil.

Note: This procedure does not extract all soil protein and should be considered as a potential indicator of the associated organic soil N pool.

Eq. (1)

$$[a * (Abs - Abs_{blank}) - b] = sample\ concentration$$

Eq. (2)

$$\left(Conc_{sample} \times 24\right) \div (Wt\ \times 1000) = g\ Protein\ kg^{-1} soil$$

Where:

a = slope of linear equation

b = y-intercept of linear equation

Abs_{blank} = Absorbance of blank (concentration of 0 on standard curve)

Abs = Absorbance of sample

$Conc_{sample}$ = Concentration of sample (result of Eq. 1)

Wt = Weight (g) of air-dried soil sample

Example:

Linear equation: y = 883.32x – 174.93

Abs_{blank} = 0.1205

Abs = 0.557

Wt = 2.99 g

$$[883.32 \times (0.557 - 0.1205) - 174.93] = 210.64$$

$$(210.64 \times 24) \div (2.99 \times 1000) = \mathbf{1.69\ g\ Protein\ kg^{-1} soil}$$

VIII. Clean up

1. Neutral pH, 20 *mM* sodium citrate solution is safe to be rinsed down the drain with copious amounts of water.
2. Autoclaved soil and sodium citrate solution can be accumulated in a container to allow soil to separate, and the sodium citrate solution may be dumped off the top and down the drain with copious amounts of water. This is to avoid clogging drains with soil. Alternatively, autoclaved soil and sodium citrate solution can be combined in the same disposal container as permanganate oxidizable carbon (POXC) solution, for citrate reduction of permanganate.
3. The BSA standard solutions and the BCA reagents (A & B individually or mixed) must be stored in a plastic container and picked up by DRS for appropriate disposal.
4. Clean the glass tubes and caps as soon as possible to avoid the soil solution from drying onto the glass. Glass tubes and caps are cleaned with 1% Alconox with a tube scrub brush, rinsed with warm water, and dried before the next use.

References:

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Thermo Scientific™ Pierce™ BCA Protein Assay Kit: https://www.fishersci.com/shop/products/pierce-bca-protein-assay/p-4531640

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